THE MECHANISMS OF CELLULAR RESPONSES DURING ENDOPLASMIC RETICULUM STRESS: THE UNFOLDED PROTEIN RESPONSE AS A METABOLIC AND CELL FATE SWITCH

by

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ABSTRACT

As the entry site into the secretory pathway, the endoplasmic reticulum (ER) is responsible for folding and processing secreted proteins. Both physiological and pathological conditions can perturb protein homeostasis in the lumen of the ER, a situation termed ER stress. The unfolded protein response (UPR) is an intracellular signaling pathway that is triggered by the accumulation of misfolded proteins in the ER, which is initiated by three ER transmembrane proteins (IRE1, PERK, and ATF6). Activation of the UPR is primarily directed towards restoring proteostasis, which is achieved through increasing the protein-folding capacity of the ER and reducing the quantity of proteins that enter the ER. If these efforts fail to rectify the condition, persistent ER stress initiates the signaling pathway resulting in cell death; however, how the UPR switches cells from adaptive programs to apoptosis is not understood well.

It has become increasingly clear that the UPR is activated by diverse physiological stimuli such as glucose, lipids, oxidative stress, and differentiation signals; thus, UPR signaling affects broad aspects of cell fate and the metabolism of glucose and lipids. In fact, altered metabolism, evasion of apoptosis, and defective differentiation are considered hallmarks of cancer, and many studies have demonstrated that ER stress and UPR activation are involved in a variety of cancers. Since the role of ER stress and the UPR in cancer is still not completely clarified, understanding the details of the UPR-

mediated cellular processes will give insight into the mechanism of cancer development and could suggest potential therapies.

In the work presented here, I focus on how glucose metabolism is changed during ER stress and the mechanism underlying this metabolic shift. I have found that ATF4, which is downstream of PERK, is important for upregulation of genes coding for glycolytic enzymes and lactate dehydrogenase, which suggests a shift in glucose metabolism toward aerobic glycolysis during ER stress. Furthermore, I investigate whether aerobic glycolysis occurring in osteoblast differentiation is regulated via the same mechanism. Lastly, I elucidate the function of the transcription factor hairy and enhancer of split 1 (Hes1) in cell fate decisions under ER stress.

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CHAPTER 1

INTRODUCTION

Overview

The endoplasmic reticulum (ER) is crucial for the proper folding and processing of proteins that are entering the secretory pathway. The ER provides a high-fidelity quality control system to ensure that only properly assembled and functional proteins are delivered to their ultimate destinations and that unfolded and misfolded proteins are retained and degraded. Various biochemical, physiological, or pathological stimuli can cause accumulation of unfolded or misfolded proteins, a condition known as ER stress. To reduce ER stress and restore homeostasis, cells trigger a complex signaling pathway referred to as the unfolded protein response (UPR).

Overall, the UPR promotes the protein-folding capacity of the ER by expanding its size and function through increased biogenesis of membrane components and transcription of chaperones and protein-modifying enzymes needed to fold proteins. Also, the UPR decreases the protein folding load on the ER by reducing general translation and enhancing ER-associated protein degradation. If the adaptive UPR fails to reestablish the ER to normality, cells commit to cell death by activating UPR-mediated cytotoxic pathways. However, it is largely unknown how, mechanistically, this important binary life or death decision is made.[1-3]

Beyond its homeostatic control of protein folding, increasing evidence has recognized important roles of the UPR in many biological functions, such as lipid and glucose metabolism, differentiation,[4-6] and inflammation.[7-10] Thus the ability to understand and manipulate how the UPR participates in multiple physiological processes is thought to provide therapeutic insights into developmental disorders as well as many ER stress-related diseases, including diabetes, neurological diseases, and cancer.[11-13]

In this dissertation, I seek to determine the mechanisms underlying novel outputs of the UPR. Specifically, I investigate the mechanism of glucose metabolism reprogramming induced by ER stress in flies and determine whether cells undergoing differentiation, previously thought to change glucose metabolism, use the same mechanism. Finally, I study how UPR signaling makes life or death decisions under ER stress.

ER Function

One of the primary functions of the ER is to fold and process secreted proteins. In mammals, most of these secreted proteins enter the ER co-translationally; ribosomes engaged in the synthesis of these proteins are targeted to the ER by a signal sequence at the amino terminus of the growing polypeptide chain (Figure 1.1). As the polypeptide chain elongates, it passes through the translocon channel into the ER lumen where the signal sequence is cleaved. Then, the protein disulfide isomerase and major chaperone BiP, both located in the ER lumen, mediate the formation of disulfide bonds and protein folding, respectively. Proteins are also glycosylated on specific asparagine residues within the ER. Correctly folded and modified proteins are available for transport to the Golgi apparatus while abnormally folded proteins are retained within the ER or degraded.[14-16]

As the most extensive membrane network, the ER contacts almost every other membrane-bound organelle in the cell, including mitochondria, the Golgi apparatus, endosomes, lysosomes, and the plasma membrane. These inter-organelle contacts allow the ER to influence a wide range of cellular processes. For example, the ER plays an

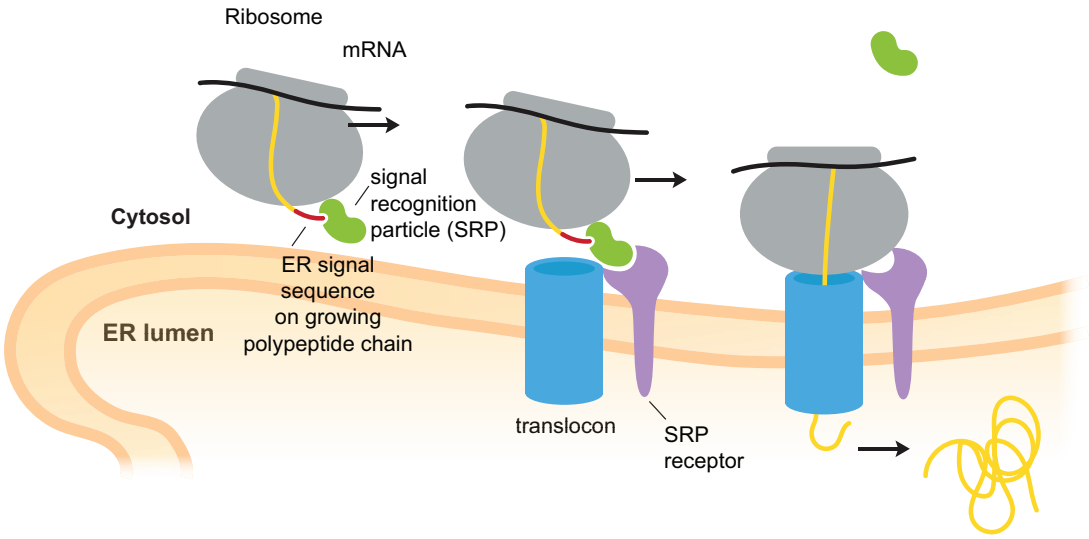

Figure 1.1 Co-translational targeting of secretory proteins to the ER. Proteins possessing an amino-terminal signal sequence are co-translationally localized to the ER. SRP binds the signal sequence as it emerges from the cytosolic ribosome. This complex docks on the SRP receptor which is embedded in the ER membrane. Following release, SRP is recycled and nascent protein chains are translated through the translocon into the lumen of the ER.

important role in synthesizing and transporting lipids to the ER-Golgi intermediate compartment so they could be distributed throughout the cell. Other functions of the ER, by operating in tandem with mitochondria, are to regulate apoptosis and homeostasis of calcium necessary for lipid synthesis, protein folding and glycosylation, apoptosis, cell cycle, and metabolism.[17-19] Because the ER can serve as the main communication centre of cellular organelles coordinating various cellular processes, the ER protein folding environment needs to be tightly regulated.

ER Stress and the Unfolded Protein Response

ER stress is caused by an imbalance between the amount of incoming unfolded proteins and the capacity of the ER to process this load. Various physiological stimuli including differentiation, hypoxia, and nutrient deprivation and pathological conditions such as diabetes, neurodegenerative diseases, and cancers can cause ER stress.[20-22] Chemicals also induce ER stress by targeting different components of the ER, such as the ER calcium ATPase pump, protein transport machinery, and protein disulfide formation.

Cells trigger the unfolded protein response (UPR) to reestablish ER homeostasis. The UPR signaling is broadly conserved across eukaryotes, and three branches of the UPR have been identified in metazoans: inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6).[3] The UPR does not only represent a stress response but is also an essential mechanism for normal development of multicellular organisms.[14,23,24] For example, mice deficient in IRE1 or in both types of ATF6α and β genes show embryonic lethality.[25-27] Perk knockout mice are viable, but exhibit severe pancreatic and skeletal defects and growth

retardation.[24,28-30]

Under normal, nonstressed conditions, these three transmembrane proteins are associated with the ER resident chaperone BiP (Figure 1.2). ER stress leads to the dissociation of BiP from the three UPR transducers, which subsequently results in their activation. Upon release from BiP, ER transmembrane Ser/Thr kinase IRE1 and PERK homooligomerize and autophosphorylate, which in turn activates them to transmit signals to the cytosol.[14,31,32] For example, phosphorylation of IRE1 elicits an endoribonuclease activity in the cytoplasmic domain, which initiates an unconventional splicing of X-box binding protein 1 (XBP1) mRNA. Dissociation of BiP from ATF6 allows translocation of ATF6 to the Golgi apparatus for processing by proteases to release the transcription activating form of ATF6.[33-37]

The UPR basically allows cells to enhance ER functions by inducing transcription of various genes, such as ER chaperones to increase the protein-folding capacity of the ER and genes involved in lipid biosynthesis to expand the volume of the ER.[30,38,39] This broad transcriptional program is mediated by XBP1, ATF6, and ATF4, which is a downstream target of PERK. To reduce the protein folding load on the ER, general protein synthesis is inhibited via phosphorylation of the cytosolic eukaryotic translation initiation factor 2α (eIF2α) at Ser51 by PERK, which halts the initiation of translation but selectively increases the translation of mRNA containing upstream open reading frames including ATF4. Regulated IRE1-dependent decay (RIDD), which degrades ER-localized mRNAs by IRE1, may also help to reduce the folding load of nascent proteins entering the ER.[33,40,41] Additionally, ER-associated protein degradation (ERAD), which removes misfolded proteins, serves as a complementary process to reduce the

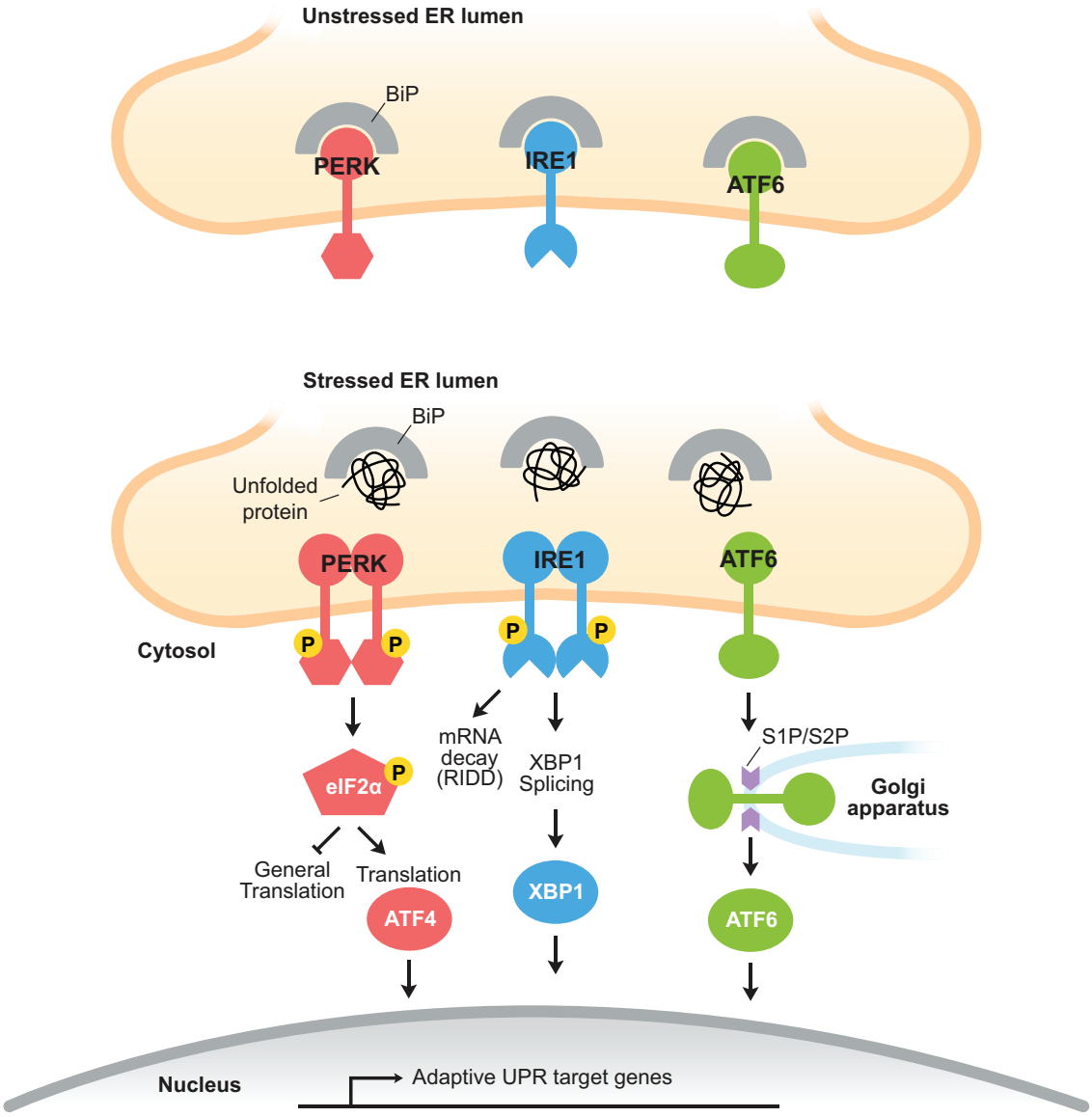

Figure 1.2 The three main signaling branches of the UPR. During unstressed conditions (top panel), the BiP chaperone binds to the luminal domain of the ER transmembrane proteins, PERK, IRE1, and ATF6, maintaining them in an inactive state. When unfolded proteins are accumulated in the lumen of the ER (bottom panel), BiP is dissociated from the UPR transducers, leading to their activation. The UPR aims to restore ER homeostasis by reducing the protein-folding load on the ER and promoting ER capacity.

unfolded protein load.

UPR-Mediated Cellular Metabolism

The first scientific question I have in Chapter 2 is how the UPR is linked to central carbon metabolism, particularly glycolysis, during ER stress. Considering that the ER is an integral contributor to cellular metabolism, such as protein synthesis and degradation, calcium homeostasis, gluconeogenesis, and lipid synthesis, UPR activation is central to regulation of both glucose and lipid metabolism.[44-46] In addition, the influence of UPR signaling on metabolic diseases, such as type 2 diabetes, insulin resistance, and non-alcoholic fatty liver disease, has been extensively documented in recent years.[2,4,47]

The role of the UPR in glucose homeostasis is relatively well established in insulin-secreting pancreatic β cells and hepatocytes that are responsive to insulin and glucagon. The importance of translational control by PERK in glucose homeostasis in β cells has been supported by studies using PERK-null mice and mice with a homozygous mutation at the eIF2α phosphorylation site (Ser51Ala). Both mice showed hyperglycemia associated with loss of pancreatic islets and defective gluconeogenesis, respectively.[35,44,48,49] Such islet dysfunctions were not reported in mice lacking GCN2 (general control nonderepressible 2). Moreover, when PERK expression was reintroduced in β cells of PERK-null mice, the mice became euglycemic and displayed normal β cell.[50,51] Transcriptional networks activated by the UPR also target expression of genes important in glucose metabolism. Genes involved in glucose output and glycogen synthesis, such as glycogen synthase 1, are targets of XBP1.[52-54] ATF4 also

augments gluconeogenesis by controlling the transcriptional activity of forkhead box protein O1 (FOXO1), which promotes hepatic gluconeogenesis and inhibits glucose utilization.[1] Although the overall requirement for ATF6 in glucose homeostasis remains unclear, it seems to reduce liver glucose production by inhibiting expression of gluconeogenic genes.[4]

UPR also regulates the process of *de novo* lipid biosynthesis, lipogenesis, to accommodate the need for ER expansion. The PERK-eIF2α and IRE1-XBP1 branches of the UPR can activate sterol regulatory element-binding protein (SREBP) 1 and 2, which are important transcriptional activators of fatty acid and cholesterol synthesis.[7,9] Unlike the other UPR branches, ATF6 seems to function in fatty acid oxidation rather than in lipid biosynthesis.[11] The regulation of lipogenesis by the UPR needs to be further investigated to better understand exactly how UPR-induced transcriptional programs are integrated into classical lipid metabolism regulation.

It is clear that activation of the UPR modulates gluconeogenesis and lipogenesis during ER stress to increase the ER capacity, although how the metabolism of glucose and lipid is differentially regulated by the UPR in different organs still requires further investigation. Moreover, there are relatively few studies connecting the UPR and glycolysis, even though relationships between glycolysis, lipogenesis, and other intermediate metabolites have been studied.

UPR-Mediated Differentiation

In Chapter 3, I address the second scientific question, whether the mechanism of ER stress-induced metabolic shift, which we found in Chapter 2, contributes to metabolic

reprogramming during osteoblast differentiation. As noted above, the UPR is required for development in multicellular organisms. It is important especially for secretory cells (Table 1.1), which expand their ER during development.[14] The role of the UPR in cell differentiation was first demonstrated by the requirement for the IRE1-XBP1 branch in the differentiation of B cells into antibody-secreting plasma cells.[17] Induction of XBP1 is required for the increased ER expansion and protein synthesis necessary for high levels of antibody production and secretion upon antigenic stimulation. It was initially thought that IRE1-XBP1 was activated by excessive protein folding-load on the ER due to massive immunoglobulin synthesis. However, the expansion of the ER was observed in B cells before the onset of immunoglobulin synthesis,[20] and mice engineered to abrogate IgM secretion showed normal XBP1 mRNA splicing.[23] These results suggest that differentiation of plasma cells is accompanied by the XBP1-mediated ER expansion, which might anticipate, rather than occur as a consequence of massive antibody production. ATF6 is also known to be activated during plasma cell differentiation, but its contributions to this process are not understood.[25] Unlike other branches of the UPR, PERK remained inactive in differentiating B cells, and mice having non-phosphorylatable eIF2α still showed B cell maturation and plasma cell differentiation.[24,29] Taken together, these findings imply that B cells activate UPR in order to accommodate sustained high levels of secretion by expanding ER rather than to limit the protein folding burden via translational control.

In contrast to differentiating B cells, all three branches of the UPR are activated during differentiation of osteoblasts. These cells secrete a large amount of type I collagen and other extracellular matrix proteins that form compact bone. However, whether UPR

Table 1.1 The role of the UPR in professional secretory cell development and differentiation.

Secretory cell types	Major function	UPR branch	Outputs	Ref.
Plasma cell	Antibody secretion	XBP1	Expansion of the ER	15, 16
			Biosynthesis and secretion of IgM	18, 19
			Transition from a membrane-bound to a secreted form of *Ig* mRNA	21, 22
		IRE1	Expression of Rag1/2 and terminal deoxynucleotidyl transferase essential for Ig gene rearrangement	14, 24
		ATF6	Its role has not been established but its activation has been observed during LPS-induced differentiation	25-27
Pancreatic β cell	Insulin synthesis	IRE1	Proinsulin biosynthesis	28, 30
		XBP1	Insulin secretion and glucose control	31, 32
		PERK	It has been hypothesized that it modulates proinsulin quality control and trafficking	34, 36
		ATF6	Regulation of glucose-mediated proliferation in β cells	38, 39
Hepatocyte	Glucose and lipid metabolism	IRE1, ATF6, PERK	Lipid and/or cholesterol biosynthesis	40, 41
		XBP1	Expression of *α-fetoprotein* and several acute phase response genes that promote hepatocyte differentiation, growth and prevent apoptosis	42, 43
Osteoblast	Secretion of the matrix for bone formation	IRE1-XBP1	Expression of *Osterix*, one of the essential transcription factors for osteoblast differentiation	45, 46
		PERK -ATF4	Expression of *Osteocalcin* (*Ocn*) and *Bone sialoprotein* (*Bsp*), which are mature osteoblast markers	4, 47
		ATF4	Type I collagen synthesis	35,48,49
		ATF6	Expression of *Ocn*	50, 51
Gastric zymogenic cell	Secretion of digestive enzymes	XBP1	Expansion of the ER Expression of Mist1, the transcription factor involved in zymogenic cell	52-54

activation is a direct consequence of increased collagen secretion or is anticipated by other differentiation signals is not clear. Bone morphogenetic protein 2 (BMP2) signaling, which is required for osteoblast differentiation, likely is involved in inducing the UPR.[14] Since both PERK- and ATF4-null mice exhibited severe skeletal defects, the role of PERK-ATF4 branch in osteoblast differentiation is relatively well studied as compared to that of other UPR branches.[33,35,37] PERK null mice showed a reduced number of mature osteoblasts and reduced type I collagen secretion,[30] which was associated with altered expression of key regulators of osteoblast development, such as *Runx2* and *Osterix.*[33] Moreover, the trafficking and secretion of type I collagen were compromised by abnormal ER retention of procollagen in PERK knockout osteoblasts, which leads to reduced mature collagen production and mineralization. While ATF4-null mice did not show a significant change in *Runx2* and *Osterix* levels, they reduced synthesis of type I collagen due to a decrease in amino acid import, a previously known function regulated by ATF4 in other cell types.[35,37] These findings indicate that both PERK and ATF4 act as novel regulators of osteoblast differentiation and function, suggesting that secretory cells customize the UPR to fit their needs.

Cell Death Pathway Induced by the UPR

The last scientific question I have in Chapter 4 is how the transcription factor Hes1 controls ER stress-mediated cell death. If the ER stress is prolonged and/or severe, and the UPR fails to restore the ER function, cells undergo apoptotic cell death by activating pro-apoptotic pathways of the UPR (Figure 1.3). Cell death under ER stress largely depends on the core mitochondrial apoptosis pathway.[44] The conformational

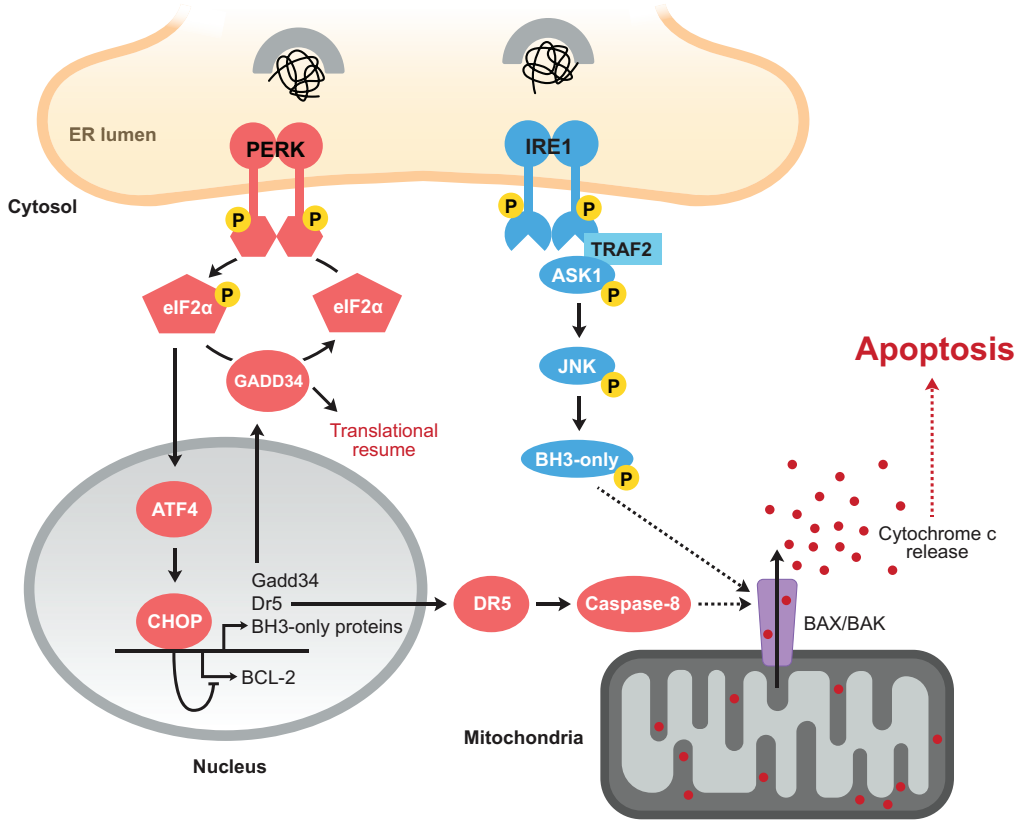

Figure 1.3 UPR-mediated apoptosis. Prolonged ER stress induces apoptosis through various pathways. Activation of the PERK-ATF4 branch induces transcription of the pro-apoptotic transcription factor CHOP. Active IRE1 recruits TRAF2 to activate ASK1 and downstream JNK, which is involved in a variety of pro-apoptotic signaling. Solid lines indicate direct regulation and dotted lines indirect mechanisms or multiple steps not shown.

activation of the proapoptotic proteins BAX and BAK by BH3 (BCL2 homology domain 3)-only proteins is a key step in this apoptosis pathway. Oligomerization of Bax and Bak is followed by permeabilization of the outer mitochondrial membrane and the release of cytochrome *c* from mitochondria. Cytosolic cytochrome *c* leads to caspase activation and subsequent cell death.

IRE1 induces apoptosis through the interaction with tumor necrosis factor receptor-associated factor 2 (TRAF2) and activating apoptosis signal-regulating kinase 1 (ASK1).[2] ASK1 enhances JUN N-terminal kinase (JNK) signaling which phosphorylates B cell lymphoma 2 (BCL2) to eliminate the anti-apoptotic effect of BCL2. Besides BCL2, JNK also phosphorylates and activates BH3-only proteins. Collectively, these changes allow oligomerization of the pro-apoptotic Bax and Bak.

Signaling through the PERK branch of the UPR also promotes apoptosis through C/EBP-homologous protein (CHOP, also known as GADD153) which is an ATF4 target gene.[44] As a leucine zipper transcription factor, CHOP has been reported to play a major role in promoting apoptosis by repressing the expression of antiapoptotic proteins such as BCL2, and inducing the expression of proapoptotic proteins, including many BH3-only proteins, death receptor 5 (Dr5), and GADD34.[2,3] Dr5 triggers caspase 8-induced BAX/BAK activation, which subsequently results in cytochrome *c* release.[5,6] Expression of GADD34 correlates with apoptosis induced by various signals, and its overexpression can initiate or enhance apoptosis.[8,10] Because GADD34 is a protein phosphatase 1 (PP1)-interacting protein, release of the translational block via dephosphorylation of eIF2α by GADD34 has been thought to cause cell death[12,13]; however, the mechanism by which GADD34 promotes apoptosis is still not clear.

Research so far has identified many components involved in the UPR-mediated cell death during ER stress; however, relatively little is known about how cells integrate the various proapoptotic signals to induce cell death. Furthermore, the mechanisms regulating the transition from an adaptive to apoptotic regime remain largely unknown.

Concluding Remarks

The widespread network of the ER in cells allows signals to reach several contacting organelles and subsequently coordinate a widespread cellular response to a particular cue. In fact, it is now becoming clear that the UPR, a signaling pathway induced by ER stress, provides a signaling framework into which other cellular pathways are intimately integrated, thereby having regulatory effect on various cellular processes, such as apoptosis, metabolism, and differentiation, other than protein folding and processing.

Although many physiological inputs that elicit UPR signaling are now known, the key question of how the UPR output alleviates ER stress by regulating a wide range of cellular functions still remain unclear. To better understand novel outputs of the UPR that are not directly linked to protein misfolding, I have used molecular biology techniques and chemical induction of ER stress. In Chapter 2, I address the question of how glucose metabolism is altered and the UPR is involved in regulation of metabolic change during ER stress. I describe the mechanism of regulation of genes encoding for the enzymes involved in central carbon metabolism during ER stress in *Drosophila* S2 cells. Based on our understanding of regulation of glucose metabolism by ER stress, I determine whether the same mechanism is exploited by cells undergoing BMP2-induced osteoblastic

differentiation in Chapter 3. Finally, I describe a new molecular mechanism controlling overall cell fate decisions under ER stress in Chapter 4.

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CHAPTER 2

DROSOPHILA MELANOGASTER ACTIVATING TRANSCRIPTION FACTOR 4 REGULATES GLYCOLYSIS DURING ENDOPLASMIC RETICULUM STRESS

G3·Genes | Genomes | Genetics

INVESTIGATION

Drosophila melanogaster Activating Transcription Factor 4 Regulates Glycolysis During Endoplasmic Reticulum Stress

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ABSTRACT Endoplasmic reticulum (ER) stress results from an imbalance between the load of proteins entering the secretory pathway and the ability of the ER to fold and process them. The response to ER stress is mediated by a collection of signaling pathways termed the unfolded protein response, which plays important roles in development and disease. Here we show that in *Drosophila melanogaster* S2 cells, ER stress induces a coordinated change in the expression of genes involved in carbon metabolism. Genes encoding enzymes that carry out glycolysis were up-regulated, whereas genes encoding proteins in the tricarboxylic acid cycle and respiratory chain complexes were down-regulated. The unfolded protein response transcription factor Atf4 was necessary for the up-regulation of glycolytic enzymes and *Lactate dehydrogenase (Ldh)*. Furthermore, Atf4 binding motifs in promoters for these genes could partially account for their regulation during ER stress. Finally, flies up-regulated *Ldh* and produced more lactate when subjected to ER stress. Together, these results suggest that Atf4 mediates a shift from a metabolism based on oxidative phosphorylation to one more heavily reliant on glycolysis, reminiscent of aerobic glycolysis or the Warburg effect observed in cancer and other proliferative cells.

KEYWORDS
unfolded protein response
endoplasmic reticulum stress
Atf4
glycolysis
metabolism

The endoplasmic reticulum (ER) is responsible for folding and processing proteins entering the secretory pathway. Because the flux of proteins through the ER varies considerably among cell types and in different conditions, cells maintain a balance between the load on the ER and its protein folding capacity. However, a number of biochemical, physiological, and pathological stimuli can disrupt this balance, resulting in ER stress. To re-establish ER homeostasis, the unfolded protein response (UPR) is activated (Walter and Ron 2011; Moore and Hollien 2012). This network of pathways up-regulates genes encoding ER-specific chaperones and other proteins involved in protein secretion (Travers *et al.* 2000) while also attenuating protein translation (Shi *et al.* 1998; Harding *et al.* 1999) and degrading certain ER-associated mRNAs (Hollien and Weissman 2006; Hollien *et al.* 2009).

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The UPR is broadly conserved across eukaryotes (Hollien 2013) and is essential for normal development in several model organisms, particularly for professional secretory cells, where it is thought to be important for the establishment and maintenance of high levels of protein secretion (Moore and Hollien 2012). It is also induced during many metabolic conditions, including diabetes, hyperlipidemia, and inflammation, and has been implicated in various cancers, especially in the growth of large tumors that rely on an effective response to hypoxia (Wang and Kaufman 2012; 2014).

The UPR is carried out by three main signaling branches. One of these is initiated by the ER transmembrane protein inositol-requiring enzyme 1 (Ire1) (Cox *et al.* 1993; Mori *et al.* 1993). When activated by ER stress, the cytosolic endoribonuclease domain of Ire1 cleaves the mRNA encoding the transcription factor Xbp1, thereby initiating an unconventional splicing event that produces the mRNA template encoding a highly active form of Xbp1 (Yoshida *et al.* 2001; Calfon *et al.* 2002). Ire1 also cleaves other mRNAs associated with the ER membrane through a pathway that is particularly active in *Drosophila* cells and that may reduce the load on the ER (Hollien and Weissman 2006; Gaddam *et al.* 2013). A second sensor of ER stress, activating transcription factor 6, is activated by proteolysis, which releases it from the ER membrane and allows it to travel to the nucleus and regulate gene expression (Haze *et al.* 1999; Wang *et al.* 2000). Finally, protein kinase RNA−like ER kinase (Perk) phosphorylates eukaryotic

initiation factor 2 alpha, leading to a general attenuation of protein synthesis as well as the translational up-regulation of certain mRNAs that contain upstream open reading frames (ORFs) in their 5′ untranslated regions (Harding *et al.* 2000). Activating transcription factor 4 (Atf4) is among those proteins that are up-regulated translationally during ER stress and regulates genes involved in protein secretion as well as amino acid import and resistance to oxidative stress (Harding *et al.* 2003).

In addition to its direct effects on the protein secretory pathway, the UPR influences several other cellular pathways, including apoptosis (Logue *et al.* 2013), inflammation (Garg *et al.* 2012), and lipid synthesis (Basseri and Austin 2012). Furthermore, the UPR (particularly the Perk/Atf4 branch) appears to have close ties to mitochondrial function. For example, knockout of Mitofusin 2, a key mitochondrial fusion protein, activates Perk, leading to enhanced reactive oxygen species (ROS) production and reduced respiration (Muñoz *et al.* 2013). Atf4 also increases expression of Parkin, which mediates degradation of damaged mitochondria, protecting cells from ER stress-induced mitochondrial damage (Bouman *et al.* 2010). Despite clear links between ER stress and mitochondria, the mechanistic relationship between the UPR and mitochondrial metabolism is not well-understood.

Here we report that the UPR in *Drosophila melanogaster* S2 cells triggers a coordinated change in the expression of genes involved in carbon metabolism. The metabolism of glucose as an energy source produces pyruvate, which can then enter the mitochondria and the tricarboxylic acid (TCA) cycle to produce reducing equivalents for oxidative phosphorylation (OXPHOS). For most cells in normal conditions, the majority of ATP is produced through OXPHOS. However, in hypoxic conditions when OXPHOS is limited, cells rely heavily on glycolysis to compensate for the decrease in ATP production and convert the excess pyruvate to lactate, which then leaves the cell (Zheng 2012). This shift from OXPHOS to glycolysis is seen in a variety of cancers even when cells have access to oxygen, an effect known as aerobic glycolysis or the Warburg effect, and is thought to be a hallmark of cancer cells (Dang 2012). Aerobic glycolysis is also becoming increasingly recognized as a metabolic signature of other cell types as well, including stem cells and activated immune cells (Fox *et al.* 2005; Rafalski *et al.* 2012).

The *D. melanogaster* estrogen-related receptor is the only transcription factor known to regulate glycolytic genes in flies (Li *et al.* 2013). Its activity is temporally regulated during mid-embryogenesis to support aerobic glycolysis during larval growth (Tennessen *et al.* 2011). Moreover, a recent study found that glycolytic gene expression under hypoxic conditions in larvae is partially dependent on *D. melanogaster* estrogen-related receptor (Li *et al.* 2013). Here, we show that the UPR transcription factor Atf4 also regulates glycolytic genes, contributing to a broad regulation of metabolic gene expression during ER stress that is reminiscent of the Warburg effect.

MATERIALS AND METHODS

Cell culture, ER stress induction, RNA interference (RNAi)

We grew *D. melanogaster* S2 cells (Invitrogen) at room temperature in Schneider's *Drosophila* media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. To induce ER stress, we treated cells with dithiothreitol (DTT, 2 mM, 5 hr) or tunicamycin (Tm, 5 μg/mL, 16 hr) unless otherwise stated.

To deplete cells of Atf4 by RNAi, we amplified a 527-nucleotide region from the coding sequence of Atf4 (also known as cryptocephal/crc, CG8669) using primers with T7 RNA polymerase sites at the 5′ ends. This amplicon has no predicted off-target 21 nt siRNA sequences, as determined using the *Drosophila* RNAi Screening Center (http://www.flyrnai.org). We used this polymerase chain reaction (PCR) product to generate double-stranded RNA (dsRNA) by *in vitro* transcription (Megascript T7 kit; Ambion). We then incubated S2 cells with 15 μg of dsRNA in serum-free media for 45 min, replaced the serum, and allowed the cells to recover for 5 d. We retreated cells with 45 μg of dsRNA and induced ER stress 1 d after the second dsRNA treatment.

RNA preparation and quantitative real-time PCR (qPCR)

We extracted total RNA from cells or decapitated flies using Trizol reagent (Invitrogen). For transfected cells, we subsequently subjected purified RNA to RQ1 RNase-free DNase I (Promega) treatment to remove residual plasmid DNA. We synthesized cDNA from 2 μg of total RNA, using a T_{18} primer and M-MuLV reverse transcriptase (NEB). We then performed qPCR using a Mastercycler ep realplex (Eppendorf) with SYBR Green as the fluorescent dye. We measured each sample in triplicate and normalized relative RNA levels to those of the housekeeping gene *Ribosomal protein L19 (RpL19)*. Sequences of all qPCR primers are listed in Table 1.

Plasmids and transfection

For Atf4 overexpression, we cloned the *D. melanogaster* Atf4 ORF downstream of the metallothionein promoter and 5′UTR, using the parent commercial plasmid pMT/V5-HisC (Invitrogen). For promoter

Table 1 Primers used for qPCR

Gene Name	Primer1	Primer2
RpL19	AGGTCGGACTGCTTAGTGACC	CGCAAGCTTATCAAGGATGG
Pfk	CTGCAGCAGGATGTCTACCA	GTCGATGTTCGCCTTGATCT
Tpi	GACTGGAAGAACGTGGTGGT	CGTTGATGATGTCCACGAAC
Ldh	GTGTGACATCCGTGGTCAAG	CTACGATCCGTGGCATCTTT
CG7430	TTTCGGCAGTAGCCAAGACT	ACCGTCTTCATGCCCATCT
l(1)G0255	CTGATGAAGATTGCCAACGA	TGCTGCCATCAGACAATAGC
CG7712	GTACCGCCAGATTCCCTACA	GCCCTGGATGAATTTTGAGA
CG4769	AAGGGCGGAGAGGATTACAT	GACCACTTGTGCCGCTTAAT
CG2968	CAAATCGATGTGCCTTCCTT	TCTATTCGGCAGCCTTGACT
BiP	ATGGTCCGACACCAATGTGCA	CCCAAATGAGTATCACCGTTG
Atf4	AGACGCTGCTTCGCTTCCTTC	GCCCGTAAGTGCGAGTACGCT
GFP	CGACCACTACCAGCAGAACA	GTAGAATCGAGACCGAGGAGAG

qPCR, quantitative polymerase chain reaction.

reporter constructs, we amplified the promoter regions of *Ldh*, *Pfk*, and *Ald* from S2 cell genomic DNA and subcloned into a vector expressing enhanced green fluorescent protein described previously (Hollien and Weissman 2006). To examine the effect of the Atf4 binding sites on the regulation these reporters, we introduced point mutations [for cyclic AMP response element (CRE)] or deleted the entire Atf4 binding motif using sequential PCR.

We transfected S2 cells with 2 μg of plasmid using Cellfectin II (Invitrogen). For polyclonal stable cell lines (as in Figure 3D and Figure 5A), we cotransfected our expression plasmids (1.8 μg) with a hygromycin or puromycin resistance plasmid (0.2 μg) and selected for resistant cells. For Atf4 overexpression studies (Figure 3D), we induced expression with $CuSO_4$ (250 μM, 36 hr) before collecting RNA samples. For promoter constructs, we treated control cells transfected with pMT-GFP with $CuSO_4$ (250 μM, 16 hr) before inducing ER stress with DTT as described above. Finally, for transiently transfected cells (Figure 5B), we allowed cells to recover overnight before inducing ER stress.

Fly strains, maintenance, and ER stress induction

We raised *Drosophila melanogaster* lines on standard medium at room temperature (~22°) unless otherwise stated. Fly stocks were as follows: w^{1118}, *hsp70*-GAL4 (*hs-GAL4*) and y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02007}attP2 (*UAS-Atf4*^{RNAi}). The *crc* (*cryptocephal*) gene encodes the *Drosophila* homolog of vertebrate Atf4 (Wu *et al.* 2007; Hewes *et al.* 2000). We obtained all fly stocks from the Bloomington Fly Stock Center (note that there was only one *Atf4* RNAi strain available). To stress flies, we grew 2- to 3-d-old males on a solid medium containing 1.3% agarose, 1% sucrose with or without Tm (10 μg/mL) for 23 hr, as described previously (Chow *et al.* 2013).

To generate *Atf4* RNAi knockdown flies, we crossed virgin *UAS-Atf4*^{RNAi} females to male *hs-GAL4*. To prevent leaky expression of GAL4 from the *heat shock* promoter, we kept the progeny at 18° until feeding them with or without Tm at room temperature as 2-d-old adults.

Lactate and oxygen consumption measurements

We plated S2 cells at 1.6×10^6 cells per well in 6-well plates in media with or without Tm (5 μg/mL, 23.5 hr). Before collecting the conditioned media from the S2 cells, we counted the number of viable cells by trypan blue (0.4%) exclusion staining. The concentration of lactate produced by cells was determined using the Lactate Colorimetric Assay Kit II (BioVision) according to the manufacturer's protocol. We measured the absorbance at 450 nm on a Synergy MX microplate reader (Biotek). Then, we divided lactate concentration by the number of cells.

For lactate measurements of flies, we homogenized at least 50 decapitated flies for each condition in PBS using a pestle. The decapitation was necessary to prevent the eye color in transgenic flies from interfering with the colorimetric lactate assay. We removed an aliquot of the homogenate and quantified total protein using the Pierce BCA protein Assay Kit (Thermo). For the remainder we heat-inactivated at 65° for 15 min and determined lactate levels as described above. We corrected the absorbance value for background by subtracting the value of same sample incubated without enzyme mix. The amount of lactate in fly samples was normalized to their protein content.

We measured the levels of dissolved oxygen in cell cultures for 5 min using Clark-type oxygen electrodes (YSI Inc.). We plotted oxygen concentration *vs.* time, and fit the data to a line to obtain the oxygen consumption rate.

RESULTS

ER stress induces a coordinated change in metabolic gene expression in S2 cells

To explore the regulation of metabolism during ER stress, we examined gene expression patterns from our previously published microarray studies of *D. melanogaster* S2 cells (Hollien and Weissman 2006). We found that the mRNA levels of most enzymes involved in central carbon metabolism changed when cells were treated with DTT (2 mM), a reducing agent that induces ER stress by disrupting disulfide bond formation within the ER. In response to DTT, genes encoding glycolytic enzymes were up-regulated whereas genes encoding TCA cycle enzymes and the respiratory chain complexes were down-regulated (Figure 1 and Supporting Information, Table S1). In addition, expression of *Lactate dehydrogenase* (*Ldh*, also known as *ImpL3* and *CG10160*), which codes for the enzyme that converts pyruvate to lactate, was dramatically increased. These changes in metabolic gene expression suggest a shift in glucose metabolism from OXPHOS to glycolysis.

To confirm these findings and to determine whether the expression changes were specific to DTT or a general response to ER stress, we treated S2 cells with another ER stress reagent, Tm (5 μg/mL), which inhibits *N*-linked glycosylation. We then monitored RNA levels of several genes over time, by qPCR (Figure 2). Consistent with the microarray results, expression of glycolytic genes and *Ldh* was increased and expression of genes encoding TCA cycle enzymes and respiratory chain complexes was decreased in response to Tm.

Atf4 regulates glycolytic genes and *Ldh* during ER stress

We next investigated which branch of the UPR signaling network is responsible for the regulation of metabolic genes during ER stress. Our array data showed that this regulation was not dependent on Ire1 or Xbp1, as depleting either of these factors by RNAi had little effect on the regulation of the metabolic genes examined (Figure 1A). We therefore tested the role of Atf4 (also known as crc), the other main transcription factor known to mediate the UPR in flies (Ryoo and Steller 2007). Targeting Atf4 in S2 cells by RNAi resulted in a reduction in Atf4 mRNA levels to 16% ± 10% compared with control cells. Knockdown of Atf4 did not affect up-regulation of *BiP* (also known as *Hsp70-3* and *CG4147*), a major ER chaperone whose up-regulation during ER stress is dependent on the Ire1-Xbp1 branch of UPR in S2 cells (Moore *et al.* 2013) (Figure 3, A and B), nor did it significantly affect the down-regulation of TCA cycle and respiratory chain complex genes (Figure 3C). However, Atf4 knockdown completely blocked induction of *Phosphofructokinase (Pfk)*, *Triosephosphate isomerase (Tpi)*, and *Ldh* by both DTT (2 mM, 6 hr) and Tm (5 μg/mL, 16 hr) (Figure 3, A and B), indicating a specific role for Atf4 in regulating glycolytic genes.

Next, we asked whether Atf4 is sufficient to up-regulate the expression of glycolytic genes. To overexpress Atf4, we stably transfected S2 cells with a plasmid expressing *D. melanogaster* Atf4 under the control of the copper-inducible metallothionein promoter. This plasmid did not include the natural 5′UTR of Atf4, whose upstream ORFs would prevent substantial translation in the absence of ER stress, but rather used the metallothionein 5′UTR. Upon addition of copper sulfate to the media (250 μM $CuSO_4$, 36 hr), transfected cells (but not untransfected control cells) displayed an increase in the expression of *Pfk* and *Ldh* (Figure 3D), similar to that induced by ER stress (Figure 3, A and B). *Tpi* mRNA levels were unchanged, however, suggesting that Atf4 is sufficient for the up-regulation of

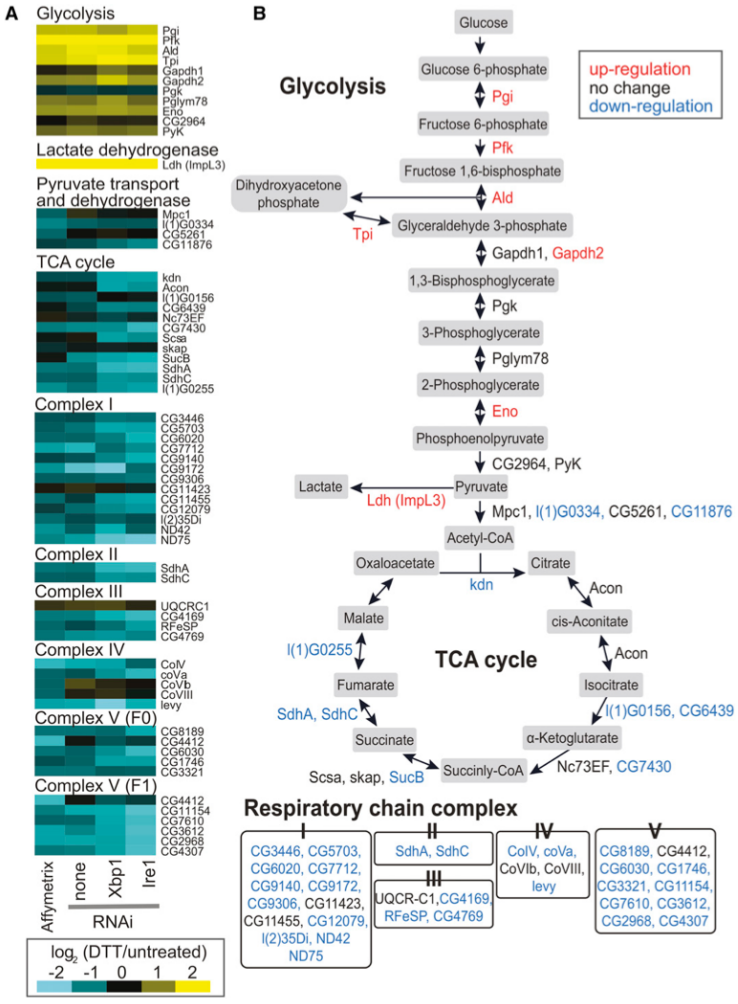

Figure 1 Microarray analysis reveals a coordinated change in the expression of metabolic genes in S2 cells treated with dithiothreitol (DTT). (A) We analyzed the expression of genes in glycolysis, the tricarboxylic acid (TCA) cycle, and the respiratory chain complex using previously published microarray data (Hollien and Weissman 2006). Briefly, S2 cells were incubated with and without DTT (2 mM, 7 hr), and relative RNA levels were measured by Affymetrix microarrays. In parallel, S2 cells were depleted of the indicated factors by RNA interference, then incubated with and without DTT (2 mM, 7.4 hr). Relative RNA levels were then measured by spotted microarray. Further details and complete data are available at the Gene Expression Omnibus, accession GPL3781 (http://www.ncbi.nlm.nih.gov/geo/). (B) Schematic diagram of glucose metabolism with expression data from (A). Red indicates mRNA levels were up-regulated at least 2-fold by DTT, blue indicates down-regulation by at least 1.5-fold, and black indicates changes between these thresholds.

a subset of the ER stress-regulated glycolytic genes. We confirmed that overexpression of Atf4 did not cause ER stress *per se* by measuring BiP mRNA levels, which were unchanged in response to $CuSO_4$ (Figure 3D). Taken together, these results indicate that Atf4 is necessary for the up-regulation of glycolytic genes and *Ldh* during ER stress, and is sufficient for the up-regulation of at least two of these genes.

Atf4 binding sites in the *Ldh* and *Pfk* promoters are important for regulation by ER stress

Atf4 is a member of the bZIP family of transcription factors, which regulate target genes through the CRE (TGACGT) (Lin and Green 1988). A recent chromatin immunoprecipitation-RNA sequencing study also identified the TT(G/T)CATCA(G/T) motif as an Atf4 binding site in mouse embryonic fibroblasts (Han *et al.* 2013). We examined the promoter regions of glycolytic genes in *D. melanogaster* (2 kb upstream and 0.5 kb downstream of the annotated transcription start sites) and found that six of the seven glycolytic genes up-regulated by ER stress contained at least one of these Atf4 binding sites. Conversely, only one of the four glycolytic genes not significantly up-regulated by ER stress contained an Atf4 binding site (Figure 4).

To investigate the importance of these Atf4 binding sites in up-regulating glycolytic genes during ER stress, we took a reporter-based

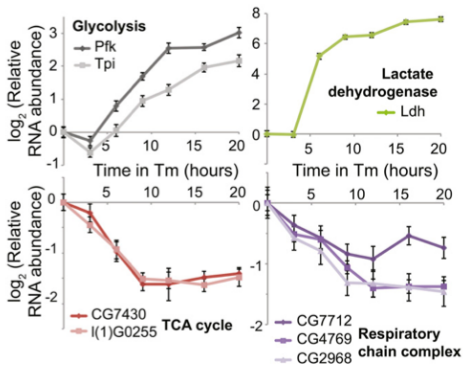

Figure 2 Metabolic gene expression is regulated by Tm in S2 cells. We treated S2 cells with Tm (5 μg/mL) and measured relative RNA levels by quantitative polymerase chain reaction. We normalized all RNA measurements to the RNA levels of *Ribosomal protein L19 (RpL19)*. Data are presented as means ± SEs of three technical replicates, and are representative of two independent experiments. *Pfk*, *phosphofructokinase*; TCA, tricarboxylic acid; Tm, tunicamycin; *Tpi*, *triosephosphate isomerase*.

approach. *Ldh*, the most highly up-regulated gene involved in the metabolic pathways studied here, contains two CREs within 2 kb upstream of its transcription start site (Figure 4). We made reporter constructs containing different lengths of the promoter region of *Ldh*, followed by the coding sequence for green fluorescent protein (GFP) (Figure 5). We transfected S2 cells with these p*Ldh*-GFP plasmids, incubated cells with and without DTT (2 mM, 5 hr), and measured the expression levels of GFP mRNA by qPCR. As a negative control, we used a plasmid expressing GFP under the control of the metallothionein promoter, and added $CuSO_4$ to cells (250 μM, 16 hr) before the addition of DTT.

We found that when we included 2 kb upstream of the transcription start site for *Ldh* in our reporter, GFP mRNA was up-regulated 12-fold during ER stress, in an Atf4 dependent manner (Figure 5A). However, when we included only 1 kb of the promoter, a region that lacks the Atf4 binding sites described above, GFP mRNA levels were not significantly changed during ER stress (Figure 5B). To test the importance of the CRE sites more specifically, we introduced into each CRE two point mutations that had been previously shown to abolish Atf4 binding in mammalian cells (Bouman *et al.* 2010). Mutating either the upstream CRE or both of the CREs in the 2 kb promoter partially blocked its regulation during ER stress (Figure 5B).

Some glycolytic genes in flies, including *Pfk*, lack CREs but contain the TT(G/T)CATCA(G/T) motif (Figure 4). To test the importance of this motif, we constructed a reporter containing 2.5 kb of the *Pfk* promoter, followed by the coding sequence for GFP. Cells stably transfected with p*Pfk*-GFP showed increased levels of GFP mRNA during ER stress, an effect that was abolished when Atf4 was depleted by RNAi (Figure 5A). Furthermore, deleting the TTGCATCAG motif in this reporter blocked GFP up-regulation by ER stress (Figure 5B).

Together, our results support a model where Atf4 regulates the expression of glycolytic genes by binding to known motifs within their promoters. However, this was not true for every promoter we tested: repeating the above reporter-based assay using 1.6 kb upstream of the *Aldolase* (*Ald*) transcription start site resulted in clear Atf4-dependent up-regulation of GFP during ER stress (Figure 5A), but mutation of the 2 CREs within this reporter had no effect on its regulation (Figure 5B). These results open up the possibility that Atf4 also regulates glycolytic genes through alternative binding sites (Fawcett *et al.* 1999; Gombart *et al.* 2007; Gjymishka *et al.* 2008; Kode *et al.* 2012; Chiang *et al.* 2013; Han *et al.* 2013) or via both direct and indirect mechanisms.

Ldh expression and lactate levels are increased in *Drosophila melanogaster* exposed to ER stress

The metabolic gene expression changes described above suggest a switch from an OXPHOS-based metabolic state to a more glycolytic one. Because OXPHOS is the main oxygen-consuming process in most cells, such a shift would be characterized by reduced oxygen consumption and an increase in lactate production, as pyruvate is converted to lactate rather than imported into the mitochondria (Wu *et al.* 2007). Surprisingly, neither oxygen consumption nor lactate concentration in the culture media was changed upon treatment of S2 cells with ER stress-inducing agents (Figure 6). Because this lack of effect might be due to the *in vitro* nature of the experimental system, we decided to test

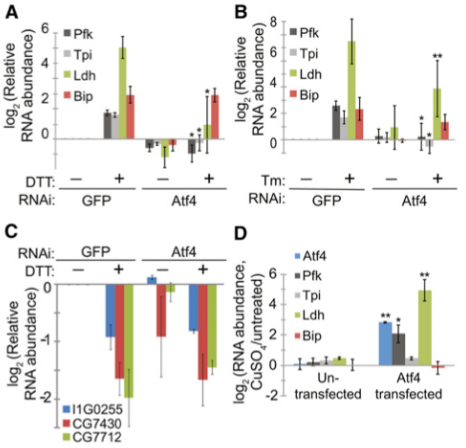

Figure 3 Atf4 is necessary and sufficient for up-regulation of glycolytic genes and Ldh. (A–B) We incubated S2 cells with dsRNA targeting either GFP (as a negative control) or Atf4, allowed cells to recover, and incubated with and without either DTT (2 mM, 6 hr, A) or Tm (5 μg/mL, 16 hr, B). We then measured the relative RNA levels for the indicated genes by qPCR. $^{*}P < 0.05$; $^{**}P < 0.005$ vs. DTT or Tm-treated control cells (GFP RNAi), Student's paired *t*-test. (C) Samples from (A) were analyzed for relative mRNA levels of TCA cycle and respiratory chain complex genes, by qPCR. (D) We stably transfected S2 cells with a plasmid expressing Atf4 under the control of a copper-inducible promoter. We incubated both untransfected and stable cell lines with and without copper (250 μM, 36 hr), then measured mRNA levels by qPCR. $^{*}P < 0.05$; $^{**}P < 0.005$ vs. untransfected control cells, student's paired *t*-test. For all panels, data are presented as means ± SDs of 3 independent experiments. Atf4, activating transcription factor 4; dsRNA, double-stranded RNA; DTT, dithiothreitol; GFP, green fluorescent protein; qPCR, quantitative polymerase chain reaction; RNAi, RNA interference; TCA, tricarboxylic acid; Tm, tunicamycin

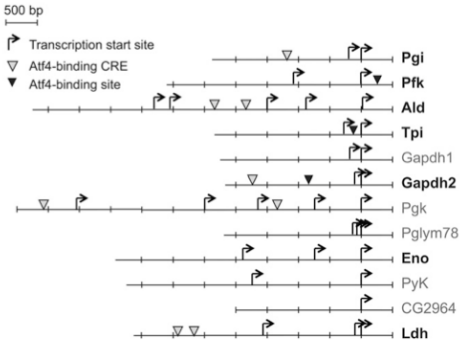

Figure 4 Potential Atf4 binding sites are found in the promoters of glycolytic genes and *Ldh*. Schematic representations of the location of putative Atf4 binding sites are shown. Arrows indicate transcription start sites as annotated in FlyBase, and arrowheads indicate consensus Atf4 binding CREs (TGACGT; gray) and the Atf4 binding motif identified in mouse embryonic fibroblasts (Han *et al.* 2013) (TT(G/T)CATCA (G/T); black). We examined 2 kb upstream and 0.5 kb downstream of all transcription start sites. Genes whose expression was up-regulated by twofold or greater by DTT are indicated in bold (see Figure 1); others were not changed during ER stress. Atf4, activating transcription factor 4; DTT, dithiothreitol; ER, endoplasmic reticulum; *Ldh*, *Lactate dehydrogenase*.

whether metabolic gene expression changes occur *in vivo* and whether they are mirrored by changes in actual metabolism.

To induce ER stress in flies, we fed *Drosophila melanogaster* strain w^{1118} media containing Tm (10 μg/mL) for 23 hr. We then isolated RNA from flies and measured the mRNA levels for several genes by qPCR. Consistent with our findings in S2 cells, flies fed Tm showed increased *Ldh* expression (Figure 7A). Levels of other glycolytic genes, however, were not significantly changed in the presence of Tm. Because *Ldh* was the most strongly up-regulated metabolic gene in S2 cells (~100 fold), and was regulated to a much lesser extent in flies (~3 fold), it is possible that this regulation occurs only in certain tissues, resulting in expression changes for other genes that were below our detection limit when whole flies were assayed.

The *Atf4* gene in flies is essential for normal development; null mutants for *Atf4* are lethal (Hewes *et al.* 2000). To examine whether *Ldh* regulation by ER stress in flies is dependent on Atf4, we therefore used the GAL4/UAS system to inducibly knockdown *Atf4* expression by RNAi. We crossed a *heatshock GAL4 (hs-GAL4)* line to a *UAS-*$Atf4^{RNAi}$. We then heat-shocked (37°, 45 min) the progeny of these crosses (*hs-GAL4* > *UAS-*$Atf4^{RNAi}$) at various times during development, but did not detect any changes in adult *Atf4* expression by qPCR. However, we saw an approximately 50% reduction of Atf4 in *hs-GAL4* > *UAS-*$Atf4^{RNAi}$ flies, compared to either parental strain, when they were continuously grown at 18° and then shifted to RT (22°) and treated with or without Tm (10 μg/mL, 23 hr) (Figure 7B). Flies depleted of Atf4 displayed a significant decrease in the up-regulation of *Ldh* during ER stress relative to the parental strains (Figure 7C). The up-regulation of BiP, in contrast, was consistent across all fly lines (Figure 7C). Thus, Atf4 up-regulates *Ldh* in flies during ER stress.

To test whether the increased expression of *Ldh* resulted in increased lactate production in response to ER stress, we measured lactate levels in extracts from flies incubated with and without Tm. Flies exposed to ER stress exhibited a ~30% increase in lactate compared with control flies, suggesting a metabolic shift to glycolysis (Figure 7D). Although analysis of lactate levels in flies depleted of Atf4 suggested that this effect was dependent on Atf4, the changes especially in *UAS-*$Atf4^{RNAi}$ flies were too small to allow for detection of significant differences between parental and progeny strains (Figure 7D). Taken together, these results suggest that flies subjected to ER stress up-regulate *Ldh* expression in an Atf4-dependent manner, and that unlike in S2 cells, this regulation results in a metabolic shift evidenced by the accumulation of lactate.

DISCUSSION

We have shown that *D. melanogaster* S2 cells subjected to ER stress up-regulate glycolytic genes and *Ldh* and down-regulate genes involved in the TCA cycle and respiratory chain complex (Figure 1 and Figure 2). Furthermore, Atf4 is responsible for the up-regulation of glycolytic genes and *Ldh* (Figure 3 and Figure 7). How TCA cycle and respiratory chain complex genes are down-regulated during ER stress requires further investigation, although the lack of effect of Atf4 depletion (Figure 3C) suggests that these are not regulated as an indirect consequence of glycolysis up-regulation.

Despite a highly coordinated change in gene expression for metabolic genes during ER stress, we did not detect any changes in

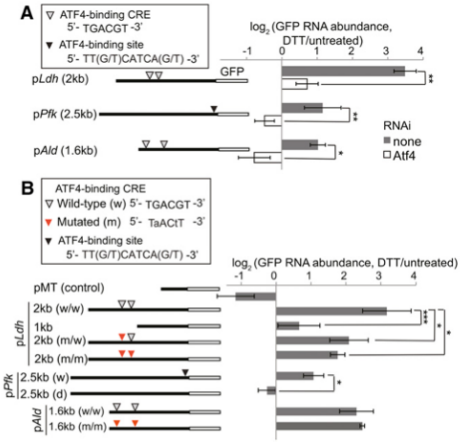

Figure 5 Atf4 binding sites within the promoters of Ldh and Pfk mediate Atf4-dependent transcriptional up-regulation. (A) We stably transfected S2 cells with the GFP reporter constructs diagrammed on the left. We then mock-treated or depleted cells of Atf4 by RNAi, incubated with and without DTT (2 mM, 5 hr), and measured relative GFP RNA levels by qPCR. (B) We transiently transfected S2 cells with reporter constructs containing wild-type or mutated promoter sequences as shown on the left. We then incubated cells with or without DTT (2 mM, 5 hr) and measured relative GFP RNA levels by qPCR. For all panels: shown are the means ± SDs of at least three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.001$, Student's paired *t*-test. Atf4, activating transcription factor 4; dsRNA, double-stranded RNA; DTT, dithiothreitol; GFP, green fluorescent protein; *Ldh*, *Lactate dehydrogenase*; *Pfk*, *phosphofructokinase*; qPCR, quantitative polymerase chain reaction; RNAi, RNA interference; TCA, tricarboxylic acid; Tm, tunicamycin

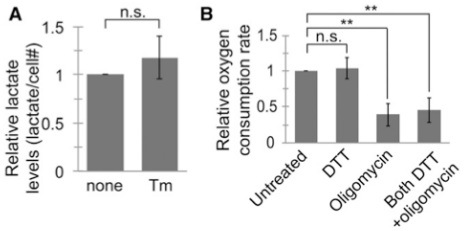

Figure 6 Lactate production and oxygen consumption are not changed in S2 cells during ER stress. (A) We measured lactate levels in the culture media of S2 cells treated with or without Tm (5 μg/mL, 23 hr). Although DTT is a typically more potent inducer of ER stress in these cells, its function as a reducing agent was incompatible with the redox-based lactate assay. We normalized the lactate concentration by number of cells and to the untreated samples. (B) We incubated S2 cells with or without DTT (2 mM, 5 hr), then added +/− oligomycin (1 μg/mL) for 10 min. We then measured the oxygen consumption rate of cells and normalized to the rate for untreated cells. For both panels, data are presented as means ± SDs of at least 3 independent experiments. ** $P < 0.005$, Student's paired *t*-test. DTT, dithiothreitol; ER, endoplasmic reticulum; n.s., not significant; Tm, tunicamycin.

actual metabolism in S2 cells (Figure 6). Because these cells have been in culture for decades and have likely been selected for rapid proliferation, it is possible that they are already undergoing some version of aerobic glycolysis, such that the underlying gene regulation during ER stress is preserved but any metabolic changes are masked. Others have also noted that S2 cells are resistant to hypoxia, and do not produce more lactate except in extreme conditions (Swiech *et al.* 2008). The increase in lactate observed through *in vivo* studies in flies subjected to ER stress (Figure 7D), however, suggests that in a more physiological setting, the gene expression changes shown here do mediate a metabolic shift toward aerobic glycolysis.

Up-regulation of glycolytic genes during ER stress has not been observed in genome-wide studies of mammalian cells (Marciniak *et al.* 2004; Hollien *et al.* 2009; Han *et al.* 2013). However, several lines of evidence suggest that mammalian cells subjected to ER stress may undergo a glycolytic shift. For example, a recent study examining human gliomas found coordinated up-regulation of UPR targets and glycolysis, which correlated with poor patient prognosis (Epple *et al.* 2013); and both ER stress (Win *et al.* 2013) and overexpression of Perk (Muñoz *et al.* 2013) have been shown to reduce mitochondrial respiration in cultured mammalian cells.

The link between ER stress and metabolism can be rationalized by the need to generate building blocks for biosynthesis of glycoproteins and lipids. Early intermediates of glycolysis are necessary for production of uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc), an important donor molecule for *N*-glycosylation of proteins in the ER. Both fructose-6-phosphate and dihydroxyacetone phosphate also are required for synthesis of glycolipids. An increased flux through glycolysis may therefore be important to support the increased production of glycerophospholipids and glycoproteins that are associated with the UPR (Sriburi 2004; Sriburi *et al.* 2006; Lee *et al.* 2008). In support of this view, glucose deprivation or inhibition of glycolysis with 2-deoxy-D-glucose induces the UPR, which contributes to cell death, especially in cancer cells (Liu *et al.* 2009; Xi *et al.* 2011; Palorini *et al.* 2013), and this effect can be rescued by UDP-GlcNAc (Palorini *et al.* 2013). The hexosamine biosynthetic pathway generating UDP-GlcNAc is also directly activated by Xbp1 (Denzel *et al.* 2014; Wang *et al.* 2014), stimulates cardioprotection during ischemia/reperfusion injury (Wang *et al.* 2014), and increases longevity in worms (Denzel *et al.* 2014).

A second, nonmutually exclusive explanation for a shift to glycolysis during ER stress is the need to limit production of ROS. Along with mitochondrial respiration, protein folding in the ER is one of the main sources of ROS (Solaini *et al.* 2010), which are produced by the normal process of disulfide bond-coupled folding (Higa and Chevet 2012). If allowed to accumulate, these ROS can cause oxidative stress and damage to cells, eventually leading to apoptosis. Several studies have confirmed that ROS are produced during ER stress (Harding *et al.* 2003; Cullinan and Diehl 2006; Tavender and Bulleid 2010), when protein folding is inefficient and more rounds of oxidation and reduction are required to fold proteins. Limiting other sources of oxidative stress, such as by down-regulating the TCA cycle and thereby restricting the flux through OXPHOS (the main source of ROS in the mitochondria), may be a way to mitigate the damage and allow cells to recover more effectively.

Finally, the advantage of the Warburg effect for tumor growth may arise from the increased rate of ATP production by glycolysis compared to OXPHOS, despite its lower efficiency of conversion (Pfeiffer *et al.* 2001). By analogy, a metabolic shift during ER stress could rapidly supply ATP necessary for protein folding and processing. Indeed, cancer cells showing elevated levels of ENTPD5, an ER

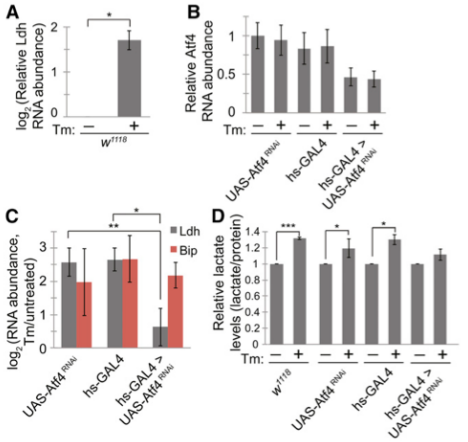

Figure 7 Flies display metabolic changes during ER stress *in vivo*. (A) We fed male *D. melanogaster* w1118 with Tm (10 μg/mL, 23 hr) to induce ER stress and measured *Ldh* mRNA levels by qPCR. (B–C) We crossed *UAS-Atf4*^{RNAi} to hs-GAL4 to obtain Atf4 knockdown flies. We stressed each strain of flies as in (A) and compared the RNA levels of *Atf4* (B), *Ldh*, and *BiP* (C) by qPCR. (D) We measured lactate levels in extracts from *D. melanogaster* fed with or without Tm as in A. Lactate concentrations were normalized using total protein concentrations. For all panels: data are presented as means ± SDs of 3 independent experiments. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$, Student's paired *t*-test. Atf4, activating transcription factor 4; ER, endoplasmic reticulum; *Ldh*, *Lactate dehydrogenase*; qPCR, quantitative polymerase chain reaction; Tm, tunicamycin.

UDPase, promotes aerobic glycolysis to increase ATP for protein *N*-glycosylation and refolding (Fang *et al.* 2010; Shen *et al.* 2011).

Overall, our results identify Atf4 as a transcriptional regulator of glycolysis during ER stress. As *Atf4* is expressed throughout fly development (Hewes *et al.* 2000), it may regulate glycolysis in other situations as well: notably, *Atf4* mutant flies are lean and have reduced circulating carbohydrates, suggesting a role in metabolism (Seo *et al.* 2009). Furthermore, because the Perk-Atf4 branch of UPR is activated during hypoxia (Blais *et al.* 2004), it will be interesting to see whether Atf4 contributes to regulation of glycolysis in other developmental, physiological (hypoxia), or pathological process during which glycolysis regulated. More broadly, because the UPR is activated in many types of cancer, its ability to regulate glucose metabolism may play a contributing role in the Warburg effect.

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CHAPTER 3

REGULATION OF AEROBIC GLYCOLYSIS AND THE UNFOLDED PROTEIN RESPONSE DURING OSTEOBLAST DIFFERENTIATION

Introduction

Glucose, a major source of cellular energy and new cell mass, is metabolized via glycolysis to pyruvate, which can enter the mitochondria and the tricarboxylic acid (TCA) cycle to produce reducing equivalents for oxidative phosphorylation (OXPHOS). The majority of ATP is produced through OXPHOS in most cells. However, under conditions of hypoxia, OXPHOS is limited and cells primarily rely on glycolysis to compensate for the decrease in ATP production, converting the excess pyruvate to lactate.[1] This shift from OXPHOS to glycolysis is seen in a variety of cancers, regardless of availability of oxygen, an effect known as aerobic glycolysis or the Warburg effect.[2]

This metabolic reprogramming also has been reported in differentiation of various kinds of cells.[3-5] Lactate dehydrogenase A (LDHA), which converts pyruvate to lactate, is a key enzyme for aerobic glycolysis. LDHA promotes differentiation of T helper 1 cells by increasing acetyl-coenzyme A to increase expression of pro-inflammatory cytokine interferon-γ.[4] Another study using a mouse bone marrow stromal cell line, ST2, provides an example of how aerobic glycolysis is regulated during osteoblast (bone forming cell) differentiation in response to WNT3A. Depletion of RICTOR, an mTORC2 component, abolished WNT3A-induced glucose consumption and lactate production. Moreover, knockdown of either RICTOR or LDHA suppressed expression of osteoblast marker genes by WNT3A, suggesting that metabolic reprogramming contributes to WNT-induced osteoblast differentiation.[5] However, the specific mechanism underlying this event and how increased glycolysis plays an important role in differentiation still remain to be elucidated.

As described in Chapter 2, during ER stress, *Drosophila melanogaster* shift their

metabolism toward aerobic glycolysis by increasing transcription of glycolytic genes and *lactate dehydrogenase (*Ldh*)*.[6] In addition to protecting cells from ER stress, the unfolded protein response (UPR) has essential roles in the development of cells specializing in secretion.[7] For example, the ER membrane is actively expanded in pancreatic acinar cells (which produces digestive enzymes), plasma cells (which secrete antibodies during an immune response), and osteoblasts (which secrete collagen to form bones) as they differentiate into highly secretory cells to promote the capacity of ER. The role of the UPR in plasma cells has been well-characterized: the IRE1-Xbp1 branch is required for expansion of the ER and antibody secretion,[8] while PERK is completely dispensable in plasma cell differentiation.[9] Unlike plasma cells, both IRE1 and PERK branches are necessary in osteoblast differentiation for increasing transcription of osteoblast-specific genes, *osterix* and *osteocalcin,* respectively.[10,11] However, the mechanism underlying ER expansion in osteoblasts is largely unknown. Given that osteoblasts rely on all three branches of the UPR during differentiation,[10-12] ER expansion in osteoblasts may proceed through a different mechanism as that in plasma cells.

In this chapter, we hypothesized that the UPR acts as a switch for aerobic glycolysis during osteoblast differentiation. Furthermore, since glycolytic intermediates are important for lipogenesis, we speculated that increased glycolysis promotes ER expansion to cope with increased production of extracellular matrix (ECM) proteins as cells differentiate into osteoblasts (Figure 3.1).

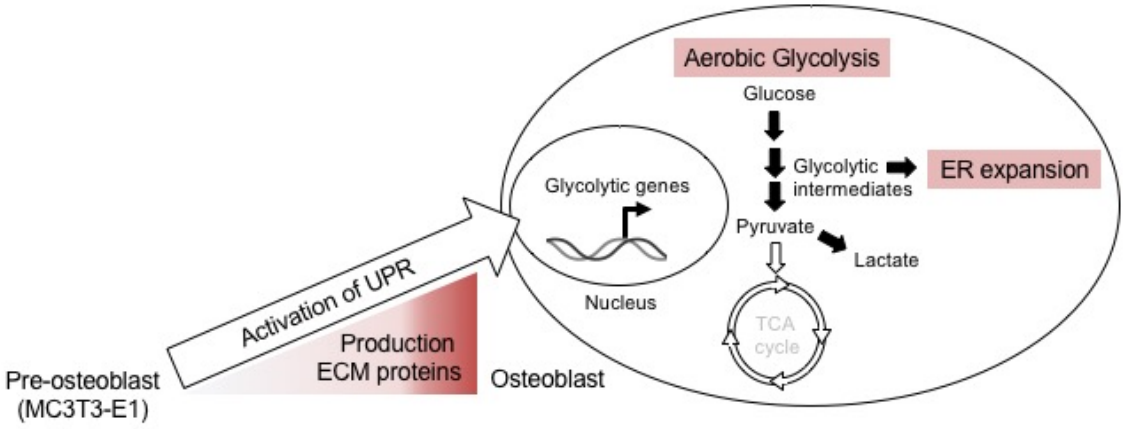

Figure 3.1. A model for how UPR links glucose metabolism to ER expansion. In response to increased demand for ECM proteins synthesis as MC3T3-E1 cells differentiate into osteoblasts, the UPR is activated. We hypothesized that the UPR induces expression of glycolytic genes that shift toward increased aerobic glycolysis. Increased production of glycolytic intermediates is important for the expansion of the ER of osteoblasts.

Materials and Methods

Cell culture and differentiation

Pre-osteoblast mouse MC3T3-E1 cells were obtained from American Type Culture Collection (Manassas, VA). They were maintained in MEMα with nucleosides and no ascorbic acid (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. We added 100 ng/mL bone morphogenic protein 2 (BMP2), 50 μg/mL ascorbic acid (AA), and 10 mM β-glycerophosphate (βGP) to cell media for indicated days to induce osteoblast differentiation.

mRNA isolation and analysis

Total RNA was isolated using Quick-RNA MiniPrep kits (Zymo Research, Irvine, CA). We then synthesized cDNA using 1 μg – 2 μg of RNA as template, a T18 primer, and MMLV reverse transcriptase (NEB). Relative mRNA levels were measured by quantitative PCR (qPCR) using the Masterplex ep realplex (Eppendorf) with SYBR green fluorescent dye. Target mRNA levels were normalized to those of ribosomal protein 19 (Rpl19) mRNA and to day 0 cells. All primer sequences are listed in Table 3.1.

RNA interference

For the Atf4 knockdown experiment, we followed RNAiMAX (Invitrogen) protocols to transfect cells with Atf4 siRNAs (Sigma). Negative siRNAs (Qiagen) were added to cells as a control. We incubated cells for 48 h before treating with and without BMP2, AA, and βGP.

Table 3.1. Primers used for qPCR and splicing assay

Name	Sequences (5′- 3′)
Rpl19	Forward: CTGATCAAGGATGGGCTGAT Reverse: GCCGCTATGTACAGACACGA
OCN	Forward: AGCAGGAGGGCAATAAGGTAG Reverse: TGCCAGAGTTTGGCTTTAGG
Osx	Forward: GACCACTTGAGCAAACATCAGC Reverse: TATCTCATCCCCAGGAAACG
Runx2	Forward: CTTCTCCAACCCACGAATGC Reverse: TATGGAGTGCTGCTGGTCTG
Wnt3a	Forward: CTGGCAGCTGTGAAGTGAAG Reverse: TGGGTGAGGCCTCGTAGTAG
Lrp5	Forward: AATCAACAAGCCACCCTCTG Reverse: GGCTCCACCAACATACTCGT
Pfk(m)	Forward: TGTCACCTCTCTGTCCTGTG Reverse: GGCTTCCCGTTCTTGTCGAT
Ldh(a)	Forward: AACATGGCGACTCCAGTGTG Reverse: ACTGTCCACCACCTGCTTGT
Xbp1	Forward: AGAAGAGAACCACAAACTCCAG Reverse: GGGTCCAACTTGTCCAGAATGC
Atf4	Forward: CGATGCTCTGTTTCGAATGG Reverse: AGAATGTAAAGGGGGCAACC

Xbp1 splicing assay

We did PCR with primers that surround the Xbp1 splice site and ran the products on a 2% agarose gel (100 V for 2 h). As a positive control, we treated MC3T3-E1 cells with 1 mM DTT for 4 h to induce ER stress.

Lactate measurement

A day before measuring lactate levels, the media from the cell culture was changed with serum free DMEM (SFM), because lactate dehydrogenase in FBS and pyruvate in our standard media interfered with the lactate assay. After changing the media, we treated cells with and without BMP2, AA, and βGP. We collected the conditioned media from the cells 12 and 24 h after replacing the media. The concentration of lactate produced by the cells was determined using the Lactate Colorimetric Assay Kit II (BioVision) according to the manufacturer's protocol. We measured the absorbance at 450 nm on a Synergy MX microplate reader (Biotek) and divided the concentration of lactate by the number of cells.

Western blotting

We lysed MC3T3-E1 cells in RIPA buffer containing protease and phosphatase inhibitors. Protein concentration was measured with the BCA method (Pierce). Proteins (10 – 20 μg for phospho-eIF2α and 40 μg for ATF4) were resolved on 4 - 12% gel (Invitrogen) and transferred onto a nitrocellulose membrane (Fischer). We blocked the membrane for 1 h at room temperature in 1% BSA in TBST containing 0.05% Tween 20, followed by an overnight incubation at 4 °C with anti-phospho-eIF2α (Ser51, ab32157,

Abcam, 1:500 or 1:1000) or anti-ATF4 antibody (C-20, Santa Cruz, 1:250). Membranes were then washed three times with TBST and further incubated with the secondary antibody, IRDye800CW conjugate goat anti-rabbit IgG (Licor, 1:10,000) in 1% BSA (TBST/ 0.05% Tween 20) for 1 h at room temperature. After being washed three times with TBST and a final wash with TBS, the blots were developed using Licor imaging software.

ER staining for analysis of ER expansion

After culturing cells in the absence and presence of BMP2, AA, and βGP, we added fresh media containing ER-Tracker blue-white DPX dye (Invitrogen, 1 μM) for 30 min at 37 °C to cells. After staining, we washed cells three times with PBS and analyzed cells stained with ER-Tracker on BD FACSAria cell sorter at the University of Utah Flow Cytometry facility.

Results

Pre-osteoblast mouse MC3T3-E1 cells increase lactate production during BMP2-induced differentiation

The classical method for osteoblast differentiation *in vitro* is based on the treatment of confluent pre-osteoblasts with BMP2, the potent regulator of osteoblast differentiation, in combination with AA and βGP.[13] AA is an essential cofactor for the hydroxylation of proline and lysine residues in collagens, which are the most abundant ECM proteins, and βGP serves as a source for the phosphate required for calcification. To determine whether we could induce osteoblast differentiation in our cell culture

system, we used pre-osteoblast mouse MC3T3-E1 cells, and three differentiation-inducing factors, BMP2, AA, and βGP. MC3T3-E1 cells treated with all three differentiation-inducing factors showed elevated levels of differentiation marker genes over time as compared to untreated or AA/βGP-treated cells (Figure 3.2), indicating BMP2 promotes differentiation of MC3T3-E1 cells. Next, we examined whether MC3T3-E1 cells shift their glucose metabolism under BMP2-induced differentiation by measuring lactate produced by the cells. We incubated cells with and without BMP2/AA/βGP for 1, 3, and 6 days. We then replaced the media with serum free media (either supplemented with and without BMP2/AA/βGP) and measured the lactate that accumulated over the following 24 h. There was no change in lactate production between untreated and BMP2/AA/βGP-treated cells at day 2 (Figure 3.3A). However, BMP2/AA/βGP-treated cells produced more lactate as compared to untreated cells at day 4 and day 7, suggesting differentiating cells rely more heavily on glycolysis.

WNT signaling is known to stimulate aerobic glycolysis in ST2 cells, a mouse bone marrow stromal cell line known to undergo osteoblast differentiation in response to WNT3A. We therefore tested whether the WNT signaling pathway is involved in the metabolic shift during BMP2-induced differentiation. We measured mRNA levels of Wnt3a and Lrp5 from MC3T3-E1 cells treated with either nothing or BMP2/AA/βGP. These mRNAs were not up-regulated in differentiating cells (Figure 3.3B). Thus, BMP2 promotes aerobic glycolysis in MC3T3-E1 cells, but does so independently of the WNT signaling pathway.

We next examined the molecular basis for the increased glycolysis. Since a coordinated metabolic gene expression change in *drosophila* S2 cells leads to increased

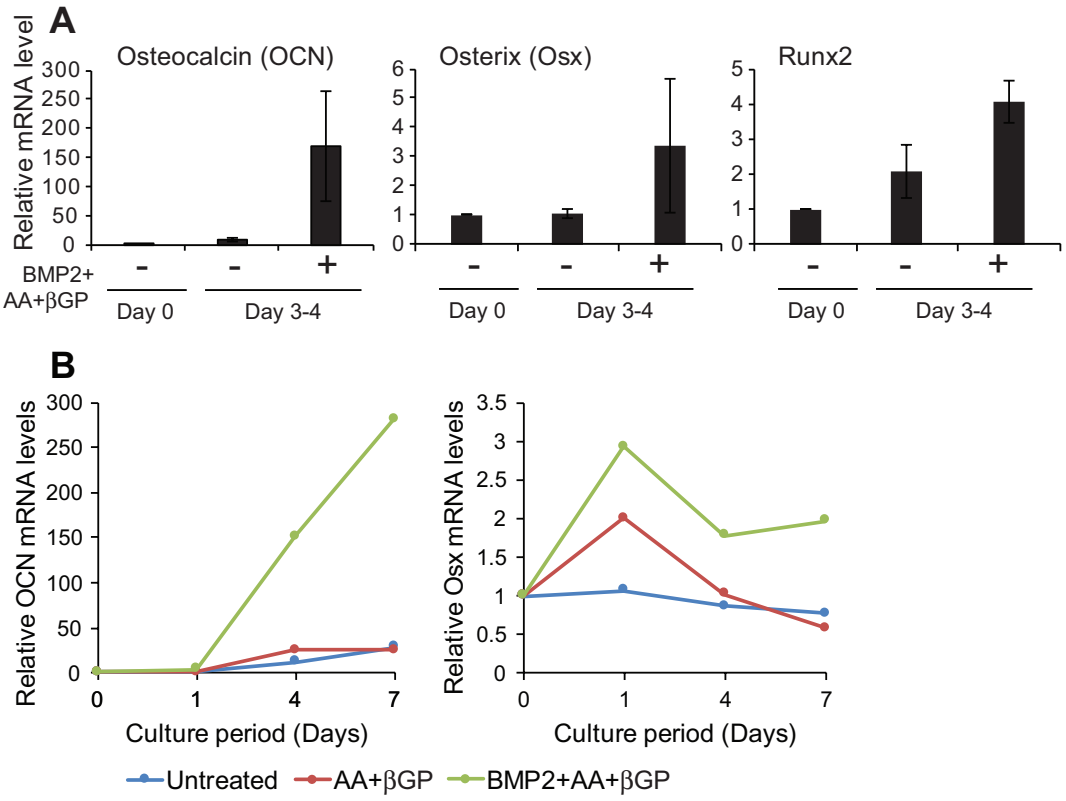

Figure 3.2. Osteoblast differentiation marker genes are up-regulated in MC3T3-E1 cells upon BMP2 treatment. (A) We cultured MC3T3-E1 cells in the absence or presence of BMP2/AA/βGP for 3 to 4 days. (B) To monitor expression of differentiation marker genes over time, we cultured cells with nothing, AA and βGP only, or BMP2, AA, and βGP for indicated days, then measured relative RNA levels by qPCR. mRNA levels were normalized to the RNA levels of Rpl19 and to day 0 cells.

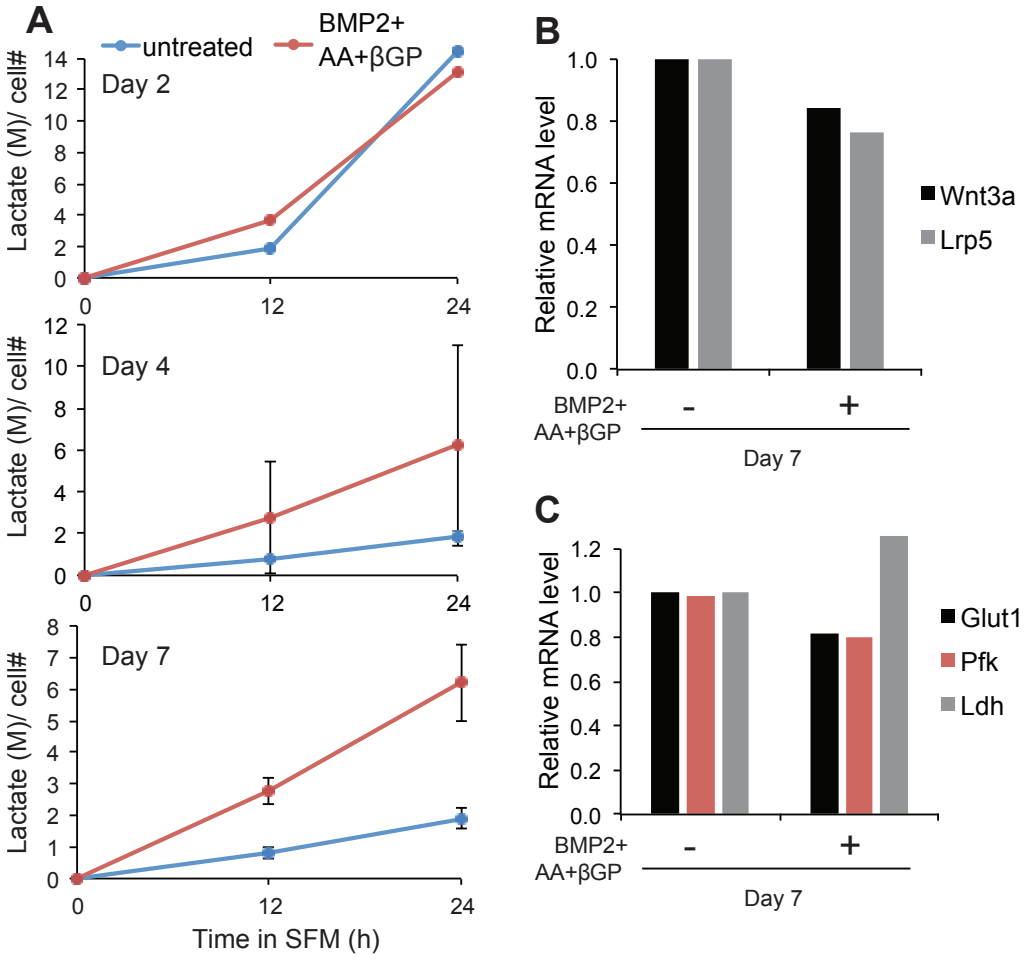

Figure 3.3. BMP2 promotes lactate production in osteoblasts. We incubated MC3T3-E1 cells with and without BMP2/AA/βGP for 2, 4, or 7 days. (A) We measured lactate levels in the culture media following incubation in SFM for indicated times. (B) We measured relative RNA levels of (B) Wnt3a and Lrp5, (C) Glut1, Pfk, and Ldh from MC3T3-E1 cells cultured in the absence and presence of BMP2/AA/βGP for 7 days by qPCR.

lactate production in response to ER stress, we decided to test if increased lactate production during BMP2-induced differentiation resulted from the increased expression of glycolytic genes or *Ldh.* However, *glucose transporter 1* (Glut1), a main glucose transporter, *phosphofructokinase* (Pfk), a rate limiting enzyme in glycolysis, and Ldh were not changed upon BMP2/AA/βGP treatment (Figure 3.3C), suggesting that the metabolic shift during osteoblast differentiation induced by BMP2 does not involve transcriptional regulation of genes encoding these glycolytic enzymes.

The UPR is not induced during BMP2-dependent osteoblast differentiation

To assess the potential involvement of the UPR in the metabolic shift during osteoblast differentiation, we first asked whether the UPR is activated in differentiating cells upon BMP2/AA/βGP treatment. Spliced Xbp1 was not observed at all in any of the experimental conditions, except the 1 mM DTT-treated control cells (Figure 3.4A). We also measured phospho-eIF2α and ATF4 protein to check PERK activity, and both levels were increased over time regardless of BMP2/AA/βGP treatment (Figure 3.4, B and C). Therefore, we had no indication that the UPR is activated during BMP2-induced osteoblast differentiation.

Expansion of the ER membrane occurs in BMP2-induced differentiation of osteoblasts

It is known that glycolytic intermediates are important for lipogenesis[14]; hence, we hypothesized that increased glycolysis during differentiation supplies the building

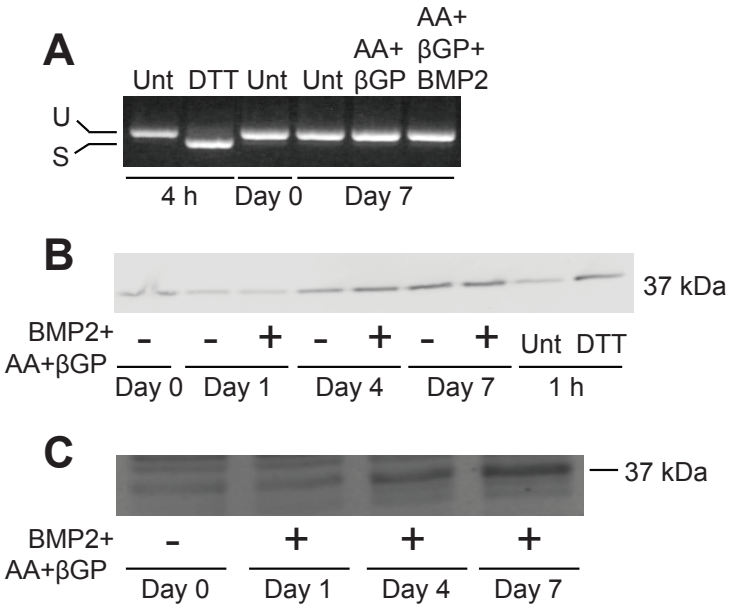

Figure 3.4. Major branches of the UPR are not activated during BMP2-induced osteoblast differentiation. We cultured MC3T3-E1 cells with indicated differentiation-inducing factors for indicated days. (A) Shown are agarose gels with the spliced (s) and unspliced (u) Xbp1 PCR products. (B) Western blot analysis of phospho-eIF2α and (C) ATF4.

blocks necessary for ER membrane expansion of osteoblasts. To test this, we first checked whether ER is expanded during BMP2-induced osteoblast differentiation by selectively staining the ER with ER-Tracker. We carried out flow cytometric analysis of ER-Tracker staining, and our preliminary result showed a 16% increase in fluorescence in BMP2/AA/βGP-treated MC3T3-E1 cells as compared to untreated cells (Figure 3.5). Since this experiment was done at a fairly early time during differentiation, we predict that extending these measurements to later time points may reveal more extended ER labeling.

Discussion

Our goal in this project was to determine whether the UPR regulates glycolysis during BMP2-induced osteoblast differentiation and examine whether this metabolic shift is important for ER membrane expansion. We demonstrated that aerobic glycolysis, as measured by lactate production, occurs during BMP2-induced osteoblast differentiation (Figure 3.3A). Unexpectedly, we found no indication that the UPR is activated by BMP2 treatment (Figure 3.4, A, B, and C). This finding was surprising because several studies have shown activation of the UPR during BMP2-induced osteoblast differentiation.[10,11] The discrepancy could be due to different controls for comparing expression of target genes and proteins; for example, phospho-eIF2α level seems to increase in BMP2/AA/βGP-treated cells when compared with day 0; however, day 4 and 7 cells treated with BMP2/AA/βGP do not have more phospho-eIF2α as compared with the corresponding cells without BMP2/AA/βGP treatment (Figure 3.4B). Increased levels of phospho-eIF2α at day 4 and 7 could be explained by a state of cell-cycle quiescence, as

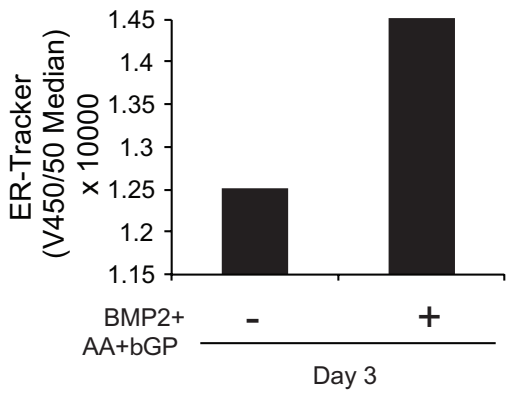

Figure 3.5. BMP2 treatment induces ER expansion of osteoblast. MC3T3-E1 cells were treated with and without BMP2/AA/βGP for 3 days, stained with the ER-specific dye ER-Tracker, and analyzed fluorescence by flow cytometry.

cells grown to confluence typically exit cell cycle, which is accompanied by active PERK. Previous studies reporting PERK activation during osteoblast differentiation did not consider this issue. Lack of spliced XBP1 in our study is more difficult to explain, but this could be due to the differences in our experimental design: for example, the types of cells and method of differentiation. Taken together, our data suggest that the metabolic shift observed in response to BMP2 is UPR-independent, and we decided to not pursue this project further at this time.

It has previously been reported that there is functional cross-talk between WNT and BMP2 signaling in mesenchymal differentiation, so BMP2 in combination with WNT3A has an inductive effect on osteoblast differentiation.[15] A recent study by Esen *et al.* showed that WNT-LRP5 signaling is involved in metabolic reprograming during osteoblast differentiation in response to WNT3A.[5] However, we did not see increased expression of *Wnt3a* and *Lrp5* upon BMP2 treatment (Figure 3.3B). Although this result indicates that WNTL-LRP5 does not regulate glucose metabolism during BMP2-induced osteoblast differentiation, it would be interesting to examine whether and how the UPR is involved in regulation of aerobic glycolysis in WNT3A-induced osteoblast differentiation.

The benefits of increased glycolysis to cells during differentiation remain to be investigated. Our results in the present study showing that metabolic shift toward glycolysis and ER expansion occur during BMP2-induced osteoblast differentiation raises the possibility that glycolytic intermediates can be bled off for anabolic synthesis of phosphatidylcholine (PtdCho), a major source of ER membrane, for ER expansion. Indeed, phosphatidic acid, which is required for PtdCho synthesis, is derived from the

glycolytic intermediate.[16] This possibility is further supported by studies showing that increased synthesis of PtdCho triggers expansion of the ER in cells undergoing ER stress to promote its capacity for protein folding.[17,18]

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CHAPTER 4

HES1 PROTECTS CELLS FROM ER STRESS-INDUCED CELL DEATH THROUGH GADD34 REPRESSION

Abstract

Disruption in ER function, termed ER stress, occurs in a variety of diseases such as neurodegenerative diseases, diabetes, and cancer. In response to ER stress, cells induce the unfolded protein response (UPR), whose activation triggers a broad transcriptional program. Those genes regulated by UPR generally encode ER-resident protein folding machinery, ER-associated protein degradation components, and other proteins of importance to ER function. We found that hairy and enhancer of split 1 (*Hes1*) mRNA is strongly stabilized during ER stress in a variety of cell types. The transcriptional repressor Hes1 is involved in the regulation of cell fate decisions as a primary target of the Notch signaling pathway; however, it does not have any obvious link to ER function. We found that Perk's ability to attenuate translation was important for *Hes1* induction and depletion of Hes1 significantly reduced cell survival in response to ER stress. ER stress-induced cell death in Hes1 knockdown cells was due to increased expression of Gadd34, a protein known to regulate apoptosis during ER stress. Thus, our results identify Hes1 as a negative regulator of apoptosis that determines overall cell fate in response to ER stress.

Introduction

As a central organelle in the secretory pathway, the endoplasmic reticulum (ER) is responsible for protein folding and posttranslational modifications. Alterations in ER functions can arise from various stimuli such as increased secretory protein synthesis and chemical treatment, which leads to the accumulation of unfolded or misfolded proteins in the ER lumen. These perturbations are referred to as ER stress. Because misfolded

proteins are potentially toxic to the cell, the ER responds to stress through the unfolded protein response (UPR) pathway.[1] This collection of signaling pathways relieves the protein-folding load on the ER by global translational attenuation and mRNA decay,[2,3] while it simultaneously increases the ability of the ER to fold proteins by up-regulating genes encoding ER-specific chaperones and proteases.[1]

The UPR is initiated by the action of three signaling proteins: PKR-like ER kinase (PERK), inositol-requiring 1 (IRE1), and activating transcription factor 6 (ATF6). Translational regulation is mainly mediated by PERK, which phosphorylates eukaryotic translation initiation factor 2 (eIF2α).[2] This inhibits general translation but promotes synthesis of protein such as ATF4, whose mRNAs contain upstream open reading frames.[4] ATF4 subsequently activates transcription of ER chaperones and genes involved in amino acid metabolism and antioxidant pathways, which are all required for ER quality control.[5] A second transducer of the UPR is IRE1, whose endoribonuclease is activated by ER stress. Active IRE1 cleaves mRNAs localized to the ER, leading to their degradation through regulated Ire1-dependent decay (RIDD).[6] It also mediates unconventional splicing of the mRNA encoding the transcription factor X-box-binding protein 1 (XBP1),[7] thereby increasing expression of many genes encoding ER chaperones and other proteins that function in the secretory pathway.[8] ATF6 is activated by proteolysis during ER stress and, along with ATF4 and XBP1, up-regulates genes necessary to reestablish protein homeostasis.[9]

In addition to its cytoprotective roles, the UPR induces apoptotic cell death if the ER stress is not mitigated.[10] Ire1 recruits TNF receptor-associated factor 2 (TRAF2), which interacts with apoptotic-signaling kinase-1 (ASK1),[11] triggering kinase cascades

that promote apoptosis.[12,13] The PERK-ATF4 branch up-regulates a pro-apoptotic transcription factor, C/EBP homologous protein (Chop), causing changes in gene expression that favor apoptosis.[14,15] For example, Chop-mediated activation of GADD34 enhances dephosphorylation of eIF2α, reversing translational attenuation, which further increases the protein-folding burden on the ER.[16] Although many studies have uncovered molecules involved in the ER stress-related apoptosis pathway, it is still unclear how the numerous signals from the stressed ER are integrated and cells decide to activate apoptosis.

In the current study, we report a novel role of hairy and enhancer of split 1 (Hes1) in cell fate decisions during ER stress. Hes1 is a member of the basic helix-loop-helix transcriptional repressors.[17] As a primary target of the Notch signaling pathway, Hes1 is known to regulate cell quiescence and proliferation in the development of multiple organs and cell types.[18] Also, nonconventional function of Hes1 has recently been highlighted by the finding that Hes1 attenuates inflammatory response via repression of chemokine transcription.[19] Here we show that Hes1 is strongly up-regulated by the UPR and affects the survival of cells exposed to chemical inducers of ER stress.

Materials and Methods

Cell culture and treatments

We cultured pre-osteoblast mouse MC3T3-E1 (American Type Culture Collection) in MEMα with nucleosides and no ascorbic acid (Invitrogen), Hek293 and 3T3 cells in DMEM (Dulbecco's modified Eagle's media) at 37°C and 5% CO_2, and *Drosophila* S2 cells (Invitrogen) in Schneider's media at room temperature. All media

were supplemented with 10% heat inactivated fetal bovine serum (FBS). To induce ER stress, we added 2 mM dithiothreitol (DTT, Sigma-Aldrich) or 2 μM thapsigargin (Tg, Sigma-Aldrich) to cell media. For inhibition of transcription or translation, we treated cells with 2 μg/mL Actinomycin D or 35 μM cycloheximide (CHX), respectively. To inhibit the integrated stress response, we added 500 nM ISRIB (kind gift from the Peter Walter lab, University of California at San Francisco) to cells for ~ 5 min before adding ER stressors. To induce HRI-mediated eIF2α phosphorylation, we treated cells with 100 μM arsenite.

RNAi

To deplete S2 cells of individual UPR transducers, we used PCR to amplify regions of cDNAs encoding Ire1, Xbp1, Atf6, Perk, Atf4, Nrf2, NFkB, using S2 cell cDNA as a template and primers containing T7 RNA polymerase sites on the 5' ends. As a control, we amplified a region of the GFP coding sequence. We then used these PCR products to generate dsRNA by *in vitro* transcription (Megascript T7 kit, Ambion). We incubated S2 cells with dsRNA in serum free media for 45 min, replaced the serum, and allowed the cells to recover for 4-5 days. We then repeated the dsRNA treatment and induced ER stress 1 day following the second dsRNA treatment. For RNAi in mammalian cells, we followed Invitrogen RNAimax guidelines for transfection of siRNAs. We combined multiple siRNAs (Qiagen) targeting each gene (Table 4.1). Negative siRNA-transfected cells were included as a control for all experiments. We subjected cells to inhibitors and/or ER stressors 48-72 h after transfection, when cells were approximately 70-80% confluent.

Table 4.1. List of siRNAs used in this study

Target gene	Species	siRNA (Cat. No.)
-	-	Negative control (SI03650325)
Perk	Mouse	Eif2ak3_1(SI00991319)
		Eif2ak3_3 (SI00991333)
	Human	EIF2AK3_1 (SI00069048)
		EIF2AK3_5 (SI02223718)
		EIF2AK3_6 (SI02223725)
		EIF2AK3_10 (SI04438224)
Upf1	Mouse	Rent1_1 (SI00228795)
		Rent1_2 (SI00228802)
		Rent1_5 (SI02719822)
		Rent1_6 (SI02744959)
	Human	UPF1_1 (SI03120432)
		RENT1_1 (SI00045598)
		RENT1_2 (SI00045605)
		RENT1_6 (SI02629963)
Hes1	Mouse	Hes1_5 (SI02667308)
		Hes1_6 (SI02686992)
		Hes1_7 (SI02708881)
		Hes1_8 (SI02732989)
	Human	HES1_2 (SI00078330)
		HES1_3 (SI00078337)
		HES1_4 (SI00078334)
		HES1_5 (SI03075016)
Gadd34	Mouse	Myd116_3 (SI00178241)
		Myd116_5 (SI02709742)
	Human	PPP1R15A_5 (SI02659125)
		PPP1R15A_6 (SI02659132)
		PPP1R15A_7 (SI03106936)
		PPP1R15A_8 (SI04439197)

Quantitative real-time PCR

We isolated mRNA using either Trizol reagent (Invitrogen) or Quick RNA MiniPrep kits (Zymo Research), and synthesized cDNA using 700 ng- 2 μg of total RNA as a template, a T18 primer, and MMLV reverse transcriptase (NEB). We measured relative mRNA abundance by qPCR using the Mastercycler ep realplex (Eppendorf) with SYBR green fluorescent dye. We measured each sample in triplicate and normalized target mRNA levels to those of ribosomal protein (Rpl19) mRNA. The primers used for qPCR are shown in Table 4.2.

Western blot

We washed Hek293 cells in PBS before lysing in RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, and 0.1% SDS) with protease inhibitors (Thermo Scientific) and phosphatase inhibitors (50 mM NaF and 0.2 mM Na-orthovanadate complexes). Protein concentration was determined using Pierce BCA protein assay kit (Thermo Scientific). We added 4x SDS loading buffer and DTT to each sample. Samples were boiled for 5 min and resolved by 4-12% NuPAGE Bis-Tris gels (Invitrogen). We transferred them to nitrocellulose membranes and probed for primary antibodies followed by a secondary IRDye 800CW antibody (Licor 926-32210, 1:10000). We scanned the blots and quantified band intensities using a Licor Odyssey imager. Each experiment was performed at least three times. Primary antibodies used for western blots were as follows: anti-Hes1 (santa cruz 25392, 1:200), anti-histone H3 (abcam 1791, 1:10000), anti-Ser51-P eIF2α (abcam 32157, 1:1000), anti-total eIF2α (abcam 26197, 1:250), and anti-Gadd34 (santa cruz 8327, 1:400).

Table 4.2. Primers used for qPCR

Name	Sequences (5´- 3´)
dRpl19	Forward: AGGTCGGACTGCTTAGTGACC Reverse: CGCAAGCTTATCAAGGATGG
mRpl19	Forward: CTGATCAAGGATGGGCTGAT Reverse: GCCGCTATGTACAGACACGA
hRpl19	Forward: ATGTATCACAGCCTGTACCTG Reverse: TTCTTGGTCTCTTCCTCCTTG
hairy	Forward: CGTGCCCGTATTAACAACTG Reverse: TCTTAACGCCATTGATGCAG
mHes1	Forward: TAACGCAGTGTCACCTTCCA Reverse: AGGCGCAATCCAATATGAAC
hHes1	Forward: CTGTCATCCCCGTCTACACC Reverse: AGGCGCAATCCAATATGAAC
mPerk	Forward: TGGACTGGTGACTGCTATGG Reverse: GGTGCTGAATGGGTAGAGGA
hPerk	Forward: CAGGCTTTTCCATCCTCATC Reverse: AACAACTCCAAAGCCACCAC
mGadd45b	Forward: GCTGTGGAGTGTGACTGCAT Reverse: ACGACTGGATCAGGGTGAAG
hGadd45b	Forward: GCCAGCTACTGCGAAGAAAG Reverse: CTCACTCCCCTTCTCCTCCT
mBim	Forward: CGACAGTCTCAGGAGGAACC Reverse: CCTTCTCCATACCAGACGGA
mDr5	Forward: AGTAGTGCTGCTGATTGGAG Reverse: CCTGTTTTCTGAGTCTTGCC
mPuma	Forward: GCCCAGCAGCACTTAGAGTC Reverse: TGTCGATGCTGCTCTTCTTG
mTrb3	Forward: GGAACCTTCAGAGCGACTTG Reverse: CCCAAAAAGTCAGGAGAAAGC
mGadd34	Forward: CTGCAAGGGGCTGATAAGAG Reverse: AGGGGTCAGCCTTGTTTTCT
hGadd34	Forward: GAGGAGGCTGAAGACAGTGG Reverse: AATTGACTTCCCTGCCCTCT
mChop	Forward: TATCTCATCCCCAGGAAACG Reverse: CTGCTCCTTCTCCTTCATGC
hChop	Forward: CAGAACCAGCAGAGGTCACA Reverse: AGCTGTGCCACTTTCCTTTC

Cell viability assay

After 4 h DTT or 7 h Tg treatment, we first removed floating dead MC3T3-E1 cells by aspirating media. We then trypsinized cells and counted the live cells using a hemocytometer. For Hek293 cells, we collected all cells after 4 h DTT treatment, then spun them down at 1000 g for 5 min to separate the dead from live cells. We resuspended cell pellets in media and counted live cells on the hemocytometer.

Results

Hairy/Hes1 mRNA is up-regulated during ER stress

Genome-wide expression analyses have previously reported that *Drosophila hairy* and mouse *Hes1* mRNA levels increase during ER stress. To confirm and extend these findings, we treated *Drosophila* S2, MC3T3-E1, and Hek293 cells with either dithiothreitol (DTT), a reducing agent that disrupts disulfide bond formation, or thapsigargin (Tg), which depletes ER calcium reserves. We then measured relative mRNA levels of *Drosophila hairy*, or mammalian *Hes1* by quantitative real-time PCR (qPCR). We found that *hairy/Hes1* mRNA levels increase during ER stress in various cells (Figure 4.1, A and B).

Since the increase in the steady-state levels of *Hes1* mRNA could result from regulation of either transcription or mRNA degradation, we next investigated whether ER stress affects the stability of *Hes1* mRNA. We treated both MC3T3-E1 and Hek293 cells with the transcription inhibitor actinomycin D, and monitored relative *Hes1* mRNA levels over time in the presence and absence of DTT. *Hes1* mRNA was rapidly degraded in both cell types in the absence of ER stress, but was strongly stabilized in the presence of DTT

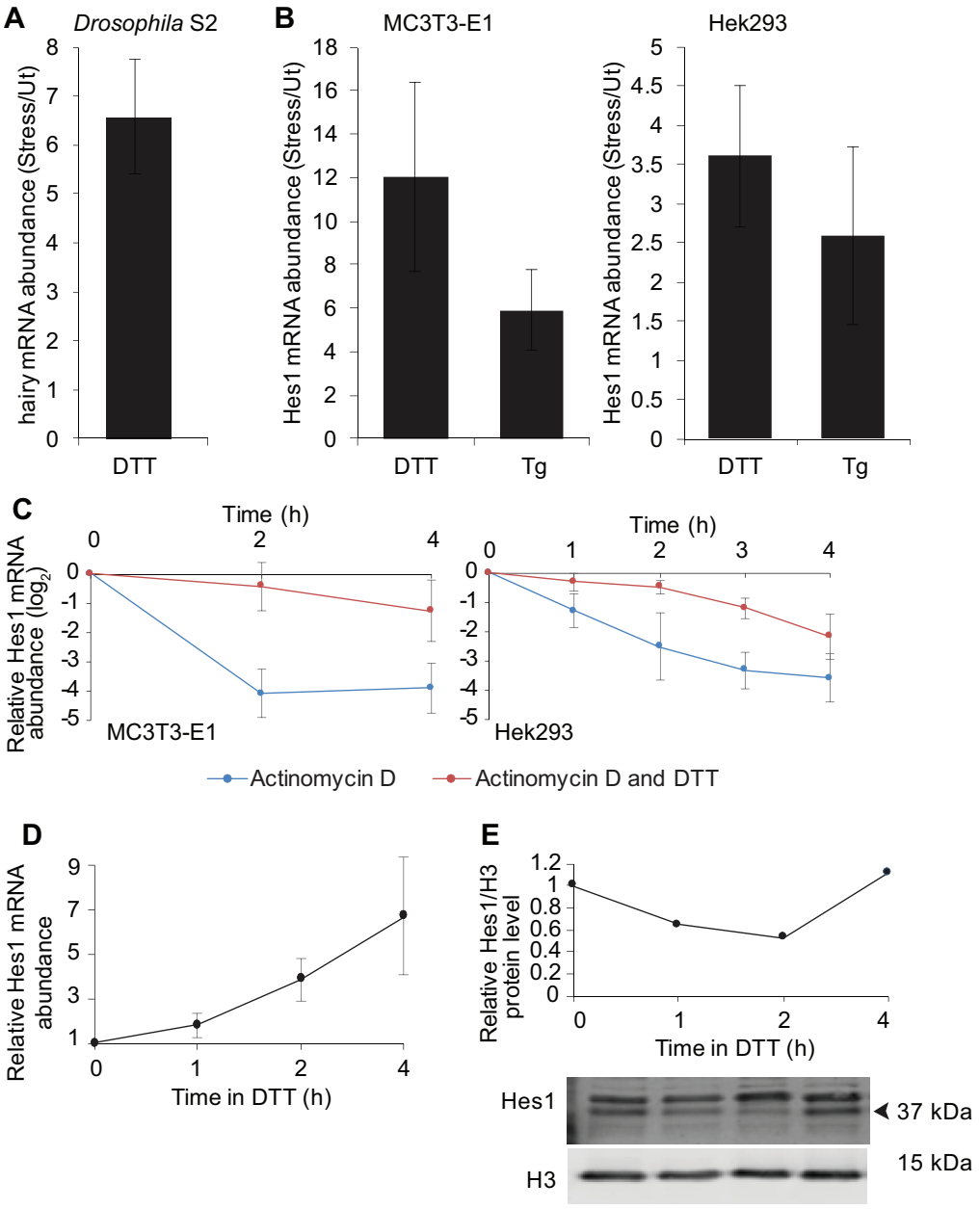


Figure 4.1. *Hes1* mRNA is increased by ER stress in various cells. We treated (A) *Drosophila* S2 cells with 2 mM DTT for 2.5 h, and (B) MC3T3-E1 and Hek293 cells with either DTT (2 mM for 5 h) or Tg (2 µM for 2 h) to induce ER stress. We then measured relative *hairy/Hes1* mRNA levels by qPCR. Data are represented as means ± SDs of at least three independent experiments. C) We treated cells with 2 µg/mL actinomycin D in the presence or absence of DTT. The means and SDs for at least two independent experiments are shown. Time course of (D) Hes1 mRNA and (E) protein in Hek293 cells exposed to DTT for indicated times. Shown are representative western blot and quantification of western blot data normalized to histone H3.

(Figure 4.1C). The magnitude of this stabilization fully accounted for the increase in *Hes1* mRNA abundance during ER stress.

Unlike the *Hes1* mRNA, Hes1 protein levels did not increase during ER stress in Hek293 cells, as determined by western blotting (Figure 4.1, D and E). Hes1p levels initially declined with DTT, returning to normal levels by 4 h. As Hes1 is reported to be a highly unstable protein, the initial decrease may be a result of UPR-mediated translational attenuation. Subsequent recovery of Hes1p levels is likely to be facilitated by the increase in mRNA levels, as eIF2α remains phosphorylated at 4 h in these conditions.

Hes1 mRNA stabilization is dependent on Perk-mediated translational attenuation

To determine which branch of the UPR signaling network is responsible for the up-regulation of *Hes1* during ER stress, we depleted each UPR transducer from *Drosophila* S2 cells using RNAi, then compared the mRNA levels of *hairy* in cells treated with and without DTT for 2.5 h (the time of maximal *hairy* induction in these cells). Perk was the sole UPR component whose depletion resulted in a loss of *hairy* up-regulation (Figure 4.2A).

To test whether mammalian Perk is necessary for *Hes1* mRNA up-regulation, we transfected both MC3T3-E1 and Hek293 cells with siRNAs targeting either Perk or a negative control sequence, and induced ER stress with DTT. Induction of *Hes1* was significantly blocked by Perk knockdown (Figure 4.2, B and C), indicating a conserved effect of Perk on *Hes1* mRNA up-regulation across species.

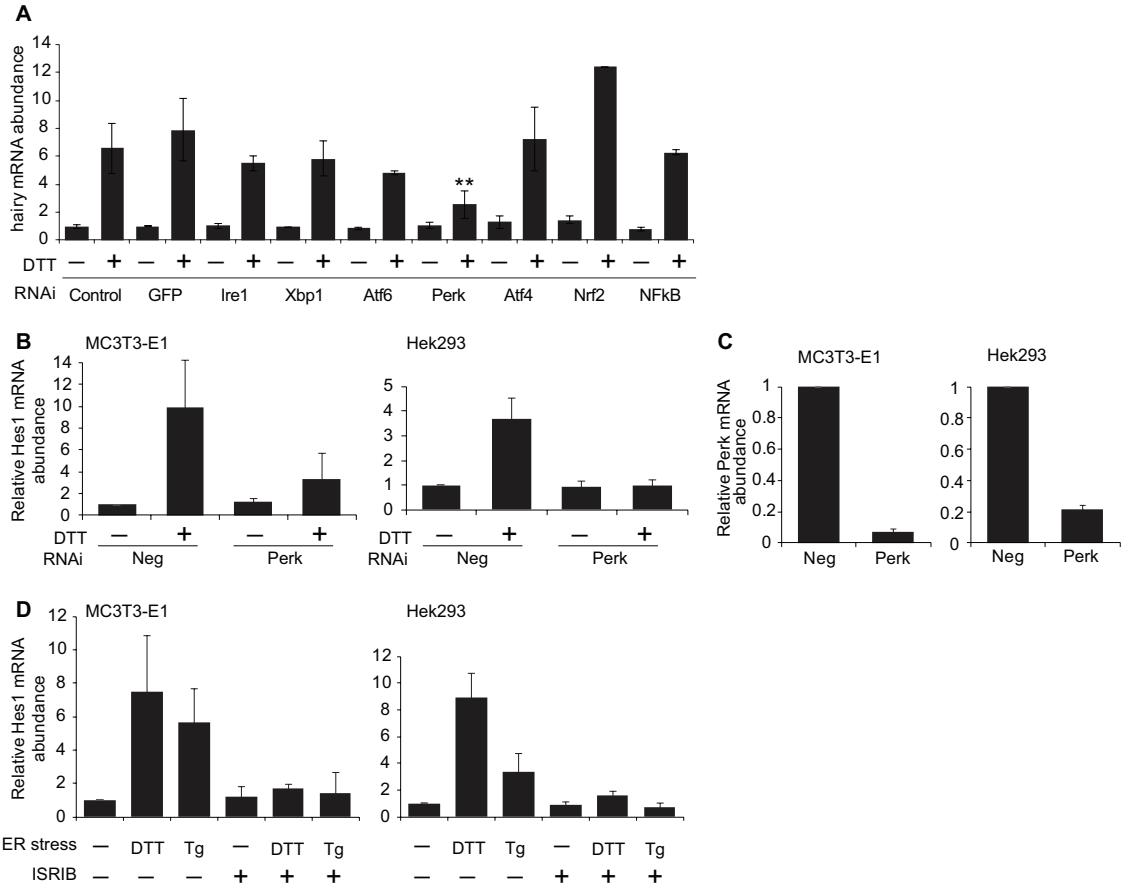


Figure 4.2. Translation attenuation mediated by Perk is important for *Hes1* regulation. (A) We incubated S2 cells with dsRNA targeting either GFP or known UPR transducers and incubated with and without DTT for 2.5 h. Control indicated untransfected cells. ** $p < 0.005$ vs. DTT-treated GFP knockdown cells, two-tailed unpaired t-test. The means and SDs for at least two independent experiments are shown. We transfected MC3T3-E1 and Hek293 cells with either Neg (negative control) or Perk siRNAs and then incubated them with or without DTT for 4 h. mRNA levels of (B) *Hes1* and (C) *Perk* were measured by qPCR. (D) We added 200 nM ISRIB, ER stressor (2 mM DTT or 2 μM Tg), or both for 2 h (4 h DTT for MC3T3-E1) to cells, and measured *Hes1* mRNA levels. Shown are means and SDs from at least three independent experiments.

Perk phosphorylates eIF2α, thereby attenuating translation initiation, as well as phosphorylating other targets such as Nrf2,[20] diacylglycerol,[21] and FOXO1.[22] To determine which aspect of Perk is important for *Hes1* mRNA regulation, we used integrated stress response inhibitor (ISRIB), a small molecule that blocks translational attenuation upon ER stress by inhibiting the downstream effects of eIF2α phosphorylation.[23] ISRIB greatly reduced *Hes1* mRNA levels induced by either DTT or Tg treatment in both MC3T3-E1 and Hek293 cells (Figure 4.2D). Overall, these results indicate that Perk-mediated translational attenuation is important for the up-regulation of *Hes1* during ER stress.

Translational attenuation is sufficient to stabilize the Hes1 mRNA

To determine whether translational attenuation is sufficient to stabilize the *Hes1* mRNA, we used two alternative approaches to attenuate translation in Hek293 cells. First, we treated cells with the translation elongation inhibitor cycloheximide (CHX). CHX treatment resulted in both an increase in the steady-state *Hes1* mRNA abundance, as well as a stabilization of the Hes1 mRNA, measured by decreased degradation following actinomycin D treatment (Figure 4.3, A and B). Second, we treated cells with arsenite, which activates the oxidative stress-responsive eIF2α kinase heme-regulated inhibitor (HRI), resulting in translation attenuation. Like ER stress and CHX, arsenite treatment led to the stabilization of the *Hes1* mRNA (Figure 4.3A), indicating that translation inhibition is sufficient for this effect.

Interestingly, arsenite alone did not increase steady-state *Hes1* mRNA levels, despite leading to levels of eIF2α phosphorylation similar to or exceeding those observed

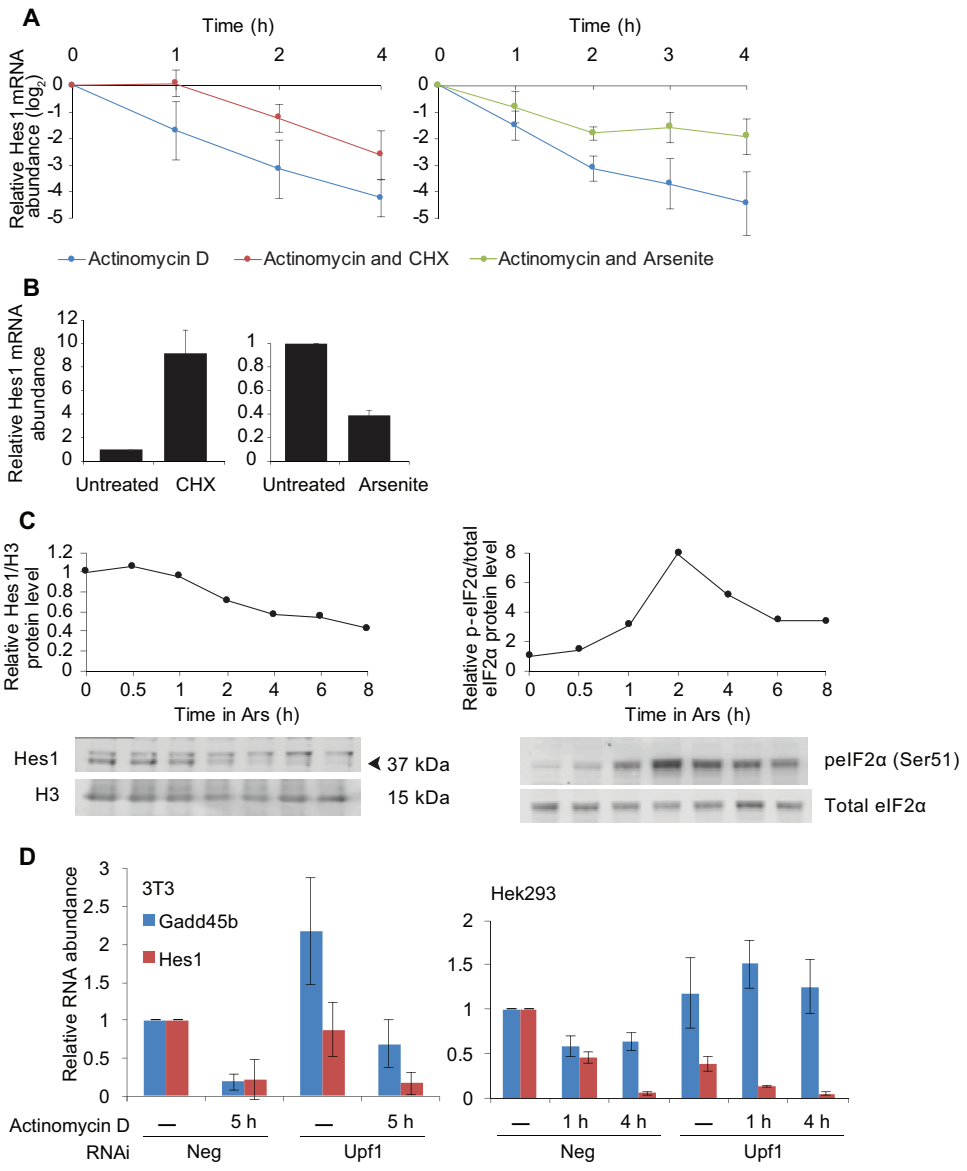


Figure 4.3. Translation inhibition is sufficient to stabilize the *Hes1* mRNA. (A) We treated Hek293 cells with 2 µg/mL actinomycin D in the presence or absence of either 100 µM Arsenite or 35 µM cycloheximide (CHX). Relative *Hes1* mRNA abundance was determined using qPCR. (B) *Hes1* mRNA levels in Hek293 cells either treated with CHX or Arsenite for 4 h. (C) Representative western blot and quantification of timecourse experiments on Hek293 cells treated with Arsenite. Hes1 and phosphorylated eIF2α (Ser51) levels were measured in whole lysates and normalized to histone H3 and total eIF2α, respectively. (D) We transfected 3T3 and Hek293 cells with either Neg or Upf1 siRNAs and then incubated them with or without actinomycin D for indicated times. We measured mRNA abundance of *Hes1* and *Gadd45b* as a control NMD target. For all panels, data are presented as means and SDs of three independent experiments.

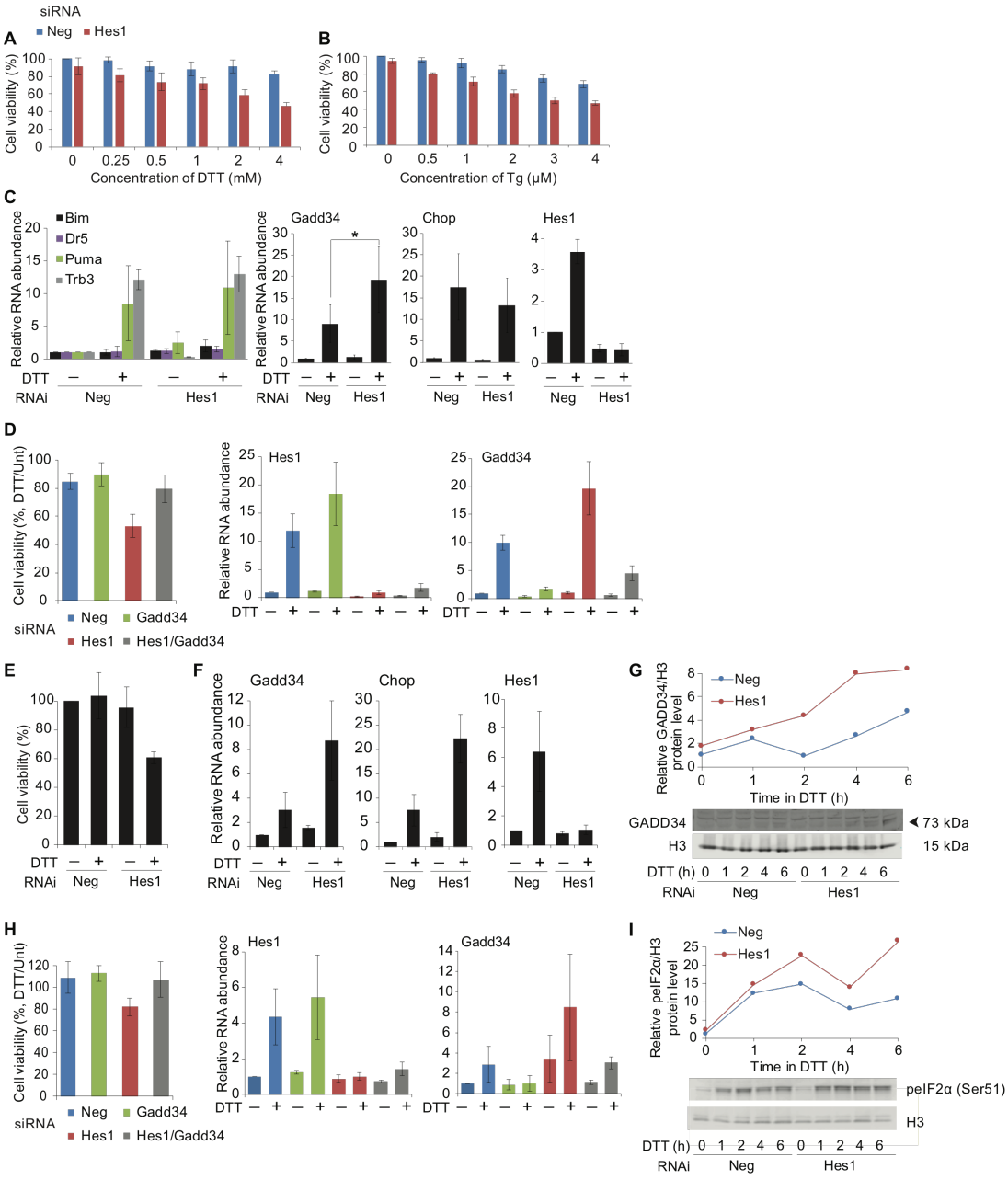
during ER stress (Figure 4.3, B and C). This suggests there are compensatory mechanisms to maintain low levels of *Hes1* mRNA during oxidative stress. Furthermore, Hes1 protein levels continued to decline over time with arsenite, rather than recover as we observed during ER stress (Figure 4.3C). This could be explained by either the lack of *Hes1* mRNA up-regulation, or by the stronger phosphorylation of eIF2α seen with arsenite compared to DTT treatment.

The fact that translation attenuation is both necessary and sufficient for stabilization of the *Hes1* mRNA suggests that this mRNA is a target of a translation-dependent mRNA decay pathway. A likely candidate for such a pathway is nonsense-mediated decay (NMD), which is known to be inhibited during ER stress.[24] However, depleting either Hek293 or mouse 3T3 cells of the core NMD factor Upf1 by RNAi did not affect the stability or steady-state levels of *Hes1* mRNA, despite having the expected effects on the known NMD target *Gadd45b* (Figure 4.3D). Thus, while *Hes1* mRNA is stabilized when translation is inhibited, it does not appear to be a direct target of NMD.

Depletion of Hes1 induces cell death in Gadd34-dependent manner in mammalian cells undergoing ER stress

To characterize the role of Hes1 during ER stress, we examined the viability of MC3T3-E1 cells transfected with either Neg or Hes1 siRNAs. Of note, Hes1 knockdown by itself did not affect cell viability in the absence of ER stressors (Figure 4.4, A and B). In contrast, DTT or Tg treatment significantly compromised cell viability in a dose-dependent manner in Hes1-depleted cells as compared to Neg siRNA-transfected cells.

Figure 4.4. Knockdown of Hes1 reduces cell viability in a Gadd34-dependent manner in response to ER stress. We performed cell viability assay on control or Hes1-depleted MC3T3-E1 cells in the presence of different concentrations of (A) DTT (4 h) or (B) Tg (7 h). (C) We compared the RNA levels of apoptotic genes induced by ER stress and *Hes1* in control and Hes1-depleted MC3T3-E1 cells. (D) Effect of double knockdown Hes1/Gadd34 on viability of MC3T3-E1 cells upon DTT treatment was determined. Hes1 and Gadd34 knockdown was confirmed by qPCR. (E) We did cell viability assay on Hek293 cells transfected either Neg or Hes1 siRNAs in the presence of DTT (4 h). (F) Samples from (E) were analyzed for relative mRNA levels of *Gadd34, Chop,* and *Hes1*. (G) Representative western blot and quantification of DTT timecourse experiments on control and Hes1-deficient Hek293 cells. Gadd34 levels were measured in whole lysates and normalized to histone H3. (H) We measured viability of Hek293 cells double knocked down Hes1/Gadd34 in the presence of DTT, and relative mRNA levels of *Hes1* and *Gadd34* by qPCR. (I) Samples from (G) were analyzed for phosphorylated eIF2α (Ser51). For all panels, data are presented as means and SDs of three independent experiments.


siRNA
Neg
Hes1
A
Cell viability (%)
Concentration of DTT (mM)
B
Cell viability (%)
Concentration of Tg (μM)
C
Relative RNA abundance
Bim
Dr5
Puma
Trb3
Gadd34
Chop
Hes1
DTT
RNAi
Neg
Hes1
D
Cell viability (%, DTT/Unt)
siRNA
Neg
Gadd34
Hes1
Hes1/Gadd34
Relative RNA abundance
E
Cell viability (%)
F
Gadd34
Chop
Hes1
G
Relative GADD34/H3 protein level
Time in DTT (h)
GADD34
H3
73 kDa
15 kDa
H
Cell viability (%, DTT/Unt)
I
Relative peIF2α/H3 protein level
peIF2α (Ser51)

Severe ER stress triggers cell death through expression of various pro-apoptotic genes.[10] We therefore measured the mRNA levels of these genes in the presence and absence of ER stress, in control and Hes1-depleted MC3T3-E1 cells. *Gadd34* was the only mRNA we tested whose expression was affected by Hes1 knockdown (Fig 4.4C). Expression of *Gadd34* was further increased during ER stress in cells lacking Hes1, consistent with a role for Hes1 in repressing the transcription of *Gadd34*.

To test whether the knockdown of Hes1 results in cell death by increasing *Gadd34*, we compared ER stress sensitivity in cells depleted of Hes1, Gadd34, and both. Co-depletion of Hes1 and Gadd34 nearly completely rescued the cell death defects caused by depletion of Hes1 alone (Figure 4.4D). Together, these data showed that the cell death-inducing downstream target of Hes1 during ER stress is Gadd34.

We confirmed these findings in Hek293 cells; depletion of Hes1 reduced cell viability upon 4h DTT treatment (Figure 4.4E), which was completely rescued by the double knockdown of Hes1 and Gadd34 (Figure 4.4H). Also, Hes1-depleted cells showed elevated levels of Gadd34 mRNA (Figure 4.4F) and protein (Figure 4.4G).

Having established that Hes1 regulates cell survival in Gadd34-dependent manner in response to ER stress, we next asked how Gadd34 induces cell death. It has been reported that Gadd34 promotes cell death by dephosphorylating eIF2α, leading to the recovery of protein synthesis and potentially exacerbating ER stress. However, we did not observe reduced phospho-eIF2α in Hes1-deficient cells as compared to control cells (Figure 4.4I), suggesting that Gadd34 can cause cell death via an alternative mechanism.

Discussion

In response to ER stress, the UPR mediates both adaptive and apoptotic pathways, depending on the duration and intensity of the stress. Many apoptotic genes involved in ER stress-induced cell death have been identified; however, the mechanism underlying the molecular switch from pro-survival phase to cell death is still largely unknown. We found that ER stress stabilizes Hes1 mRNA (Figure 4.1D), whose important function during development is known to determine cell fates. During ER stress, Hes1 functions as a negative regulator of cell death, potentially by repressing the pro-apoptotic Gadd34.

Our data indicate that Perk, by attenuating translation during ER stress, stabilizes the *Hes1* mRNA (Figure 4.2, B and D), an effect that is mimicked by CHX- and arsenite-mediated translation inhibition (Figure 4.3 A and B). The mechanism by which reduced translation affects Hes1 mRNA stability requires further investigation, although the lack of effect of Upf1 depletion suggests that *Hes1* is not a target of NMD (Figure 4.3D). *Hes1* is regulated by microRNAs such as miR-23 and miR-9 during neuronal differentiation,[25,26] and it is possible that miRNAs regulate Hes1 during ER stress as well. Consistent with this idea, the Perk branch of the UPR has been linked to miRNAs favoring cell survival.[27] For example, Perk-dependent expression of miR-211 suppresses *Chop* expression at early phases of ER stress.[28]

We found that Hes1 protects cells from ER stress-induced cell death, possibly through transcriptional repression of the pro-apoptosis gene *Gadd34* (Figure 4). Expression of *Gadd34* is induced by Atf4 and Chop, which are also downstream targets of Perk during ER stress. Because Atf4-Chop and Hes1 are each regulated by Perk but have opposite effects on *Gadd34* expression, the balance and timing of these branches of

the UPR are likely important in deciding whether cells live or die during ER stress.

Although the role of ER stress in cancer is complicated, many components of the UPR have been implicated in cancer development.[29] Intriguingly, a growing number of studies has reported that cancer cells show elevated levels of Hes1 as compared to non-malignant cells.[30,31] Moreover, high expression of *Hes1* mRNA is associated with poor prognosis in colorectal cancer.[32,33] Therefore, it will be interesting to see whether tumors with increased levels of *Hes1* are more resistant to apoptosis than tumors with low levels of *Hes1*. Our findings that Perk enhances *Hes1* mRNA stability, which prevents apoptosis during ER stress by repressing *Gadd34*, could have potential implications in cancer therapies.

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CHAPTER 5

SUMMARY AND CONCLUSION

The UPR is a complex signal transduction pathway that has diverse effects to recover ER function beyond its ability to maintain protein homeostasis during ER stress. In addition to its direct impacts on the protein secretory pathway, increasing numbers of studies are investigating the influence of the UPR on other cellular processes including inflammation, lipid synthesis, and apoptosis. In this dissertation, I have discussed two such processes, carbon metabolism and apoptosis. Here I summarize my main findings and discuss further their implications and possible future directions.

Does ATF4 Cooperatively Work to Regulate Glycolytic Genes in ER Stress and Development?

We found that ER stress induces a coordinated change in the expression of genes involved in central carbon metabolism that is highly reminiscent of the Warburg effect (Chapter 2). Genes encoding enzymes that carry out glycolysis and lactate conversion from pyruvate were upregulated while genes encoding proteins in the TCA cycle and respiratory chain complexes were downregulated in *Drosophila* S2 cells treated with ER stressors. We identified a second transcription factor, ATF4, involved in regulation of glycolytic genes and *lactate dehydrogenase* (*Ldh*) in flies during ER stress. It has been reported that the *Drosophila melanogaster* estrogen-related receptor (dERR), which was the only transcription factor known to regulate glycolytic genes in flies, directs a metabolic switch that supports larval growth.[1] ATF4 is also known to be important in regulation of developmental timing and metamorphosis[2] and null mutants for *Atf4* are lethal.[3] Consistent with these findings, we found that ATF4 depletion at early stages of fly development was lethal. Therefore, it is possible that metabolic reprogramming in

Drosophila mid-embryogenesis is accomplished by ATF4 branch of the UPR as in ER stress.

Moreover, we found that ATF4 is sufficient to induce a subset of glycolytic genes, as overexpression of ATF4 led to increased expression of *phosphofructokinase* (*Pfk*) and *Ldh* but not *triosephosphate isomerase* (*Tpi*). This result raises the possibility of collaborative role of ATF4 in glycolytic gene regulation with another transcription factor. In fact, *Tpi*, whose expression was not increased by ATF4 overexpression, has putative binding sites for dERR as well as ATF4 within the promoter,[4] supporting the idea that ATF4 may regulate transcription of some glycolytic genes with dERR under ER stress.

How is the TCA Cycle Controlled by the UPR?

Unlike regulation of glycolytic genes and *Ldh*, down-regulation of the TCA cycle and respiratory chain complex genes is conserved in mammalian cells during ER stress (data not shown). However, the mechanism behind down-regulation of these genes remains unclear, in part because the known transcription factors that mediate the UPR do not appear to affect regulation of these genes. Previous work in *D. melanogaster* adapted to hypoxia shows that hairy (*D. melanogaster* homologue of Hes1) represses several TCA cycle genes under hypoxia.[5] Also, the *hairy* loss-of-function mutants losing suppression of TCA cycle genes showed reduced survival rate under hypoxia, highlighting the importance of metabolic regulation by hairy to survival during hypoxia. Interestingly, we found that expression of *hairy* is increased in response to ER stress (Chapter 4). Taken together, down-regulation of TCA cycle genes may be mediated by transcriptional repressor hairy during ER stress. While technical problems prevented us

from testing this directly, our preliminary data obtained in mouse cells indicated that depletion of Hes1 (mammalian homologue) affects down-regulation of one TCA cycle gene, *isocitrate dehydrogenase 3 (Idh3a)*, in response to ER stress.

Alternatively, reduced expression of some TCA cycle genes can be achieved through reducing mRNA stability rather than transcriptional control. We previously found that when we inhibited transcription with actinomycin D (Act D) in the absence and the presence of ER stressor DTT in S2 cells, the relative transcript levels of TCA cycle enzymes were stable in Act D treated cells, but significantly decreased in both Act D and DTT treated cells (data not shown). This raises a possibility that the reduced level of TCA cycle genes results not from a downregulation of their transcription, but from an increase in their decay rates. Consistent with this hypothesis, our preliminary results using an assay based on a reporter plasmid showed that *CG7430*, a gene encoding one of the TCA cycle enzymes in α-ketoglutarate dehydrogenase complex, is degraded under ER stress. This reporter plasmid expresses the coding sequence and 3' untranslated region (UTR) of *CG7430* under the control of the copper-inducible *Drosophila* metallothionein promoter (not conditioned to endogenous factors), and thus removal of copper from the media allows for measurement of mRNA degradation. By using this reporter assay for other TCA cycle and respiratory chain complex genes, we will confirm whether the increase in mRNA decay is a common mechanism of regulation for these genes. Furthermore, it is of interest whether TCA cycle and respiratory chain complex genes are degraded locally on the cytoplasmic surface of the mitochondria, since it has been reported that numerous mRNA encoding mitochondrial proteins are closely associated with mitochondria.[6,7] Since we knew that mitochondrial-to-nuclear DNA ratio,

an indicator of mitochondrial mass, was not changed under ER stress (data not shown), this rules out the possibility that global downregulation of TCA cycle and respiratory chain complex genes under ER stress is due to degradation of mitochondria, a process called mitophagy.

How Do Cells Balance Between Adaptive and Apoptotic Responses to ER Stress?

One important function of the UPR pathway is to induce apoptotic cell death if ER stress is severe or prolonged. Although many proteins have been identified in the process of ER stress-induced apoptosis, the precise mechanism by which cells switch to the apoptotic pathway from an adaptive pathway in response to irreversible ER stress is still not clear. We found a novel mechanism regulating apoptosis during ER stress; ATF4 and Hes1 regulated by PERK have opposite effects on the regulation of the apoptotic gene *Gadd34* to determine whether cells die or not (Chapter 4). While there has been evidence that expression of GADD34 correlates with apoptosis induced by ER stress[8] and ATF4 is required for transcription of *Gadd34,*[9] this work was the first to demonstrate that expression of *Gadd34* is negatively regulated by the transcription repressor Hes1. Although the detailed mechanism underlying regulation of *Hes1* by ER stress remains to be further investigated, we showed that translation attenuation mediated by PERK is important for *Hes1* mRNA stabilization.

Based on these results, we hypothesize that *Gadd34* levels, which are controlled by ATF4 and Hes1 through PERK, are key to determining overall cell fate. Similar to the contribution of GADD34 on ER stress-induced apoptosis, death receptor 5 (DR5) has

been also known to act as a gauge for persistence of ER stress by integrating opposing UPR signals; the PERK branch induces *Dr5* transcription, whereas IRE1 degrades *Dr5* mRNA.[10] Elevated DR5 results in caspase 8-mediated apoptosis in response to ER stress, but *Dr5* mRNA was not increased during ER stress in our experimental conditions. Therefore, it will be interesting to monitor ER stress-induced apoptosis and the relative kinetics of regulation of *Gadd34* and *Dr5* to study how these pathways function to commit the cell to death.

How Does GADD34 Induce Apoptosis During ER Stress?

While there have been reports that GADD34 leads to apoptosis via premature dephosphorylation of eIF2α, which in turn leads to increased protein synthesis exacerbating ER stress,[11-14] it is still not completely understood how GADD34 mediates apoptosis. We showed that cells depleted of Hes1 increased GADD34 and apoptotic cell death in response to ER stress, but they did not show reduced levels of eIF2α phosphorylation. In fact, GADD34 have a number of interacting proteins and dephosphorylate them under various types of cellular stress.[15-17] We are currently investigating whether phosphorylation levels of any potential target proteins would be affected by Hes1 knockdown during ER stress. Another possibility is that GADD34 regulates cell viability through regulation of transcription of p21, a cyclin-dependent kinase inhibitor. Although it is not known whether GADD34 acts as part of a transcription factor complex, it interacts with zinc-finger transcription factor, which is known to bind to p21 promoter.[18] Also, recent study showed that the GADD34 mutant, which lacks the binding domain for protein phosphatase 1, had a reduced ability to inhibit

cell viability and enhance p21 expression as compared to the wild-type GADD34.[19]

Cytoprotective Functions of PERK May Contribute to Cancer Progression

The work described here is important not just for our understanding of basic UPR biology, but also for our ability to understand and treat diseases such as cancer. During tumorigenesis, the high proliferation rate of cancer cells requires increased activities of the ER to satisfy their demands for increased synthesis of proteins and lipids. Various cancer types showed evidence of ER stress and UPR activation[20]; thus the UPR, especially PERK branch, has received considerable attention as a promising target of anti-cancer therapies.[21] There is evidence that PERK promotes cancer development as it enhances angiogenesis[22] and autophagy[23] and limits oxidative DNA damage.[24] However, considering that PERK induces both adaptive and apoptotic responses depending on the intensity and duration of the stress, it is no surprise that PERK could serve as cytoprotective or cytotoxic function in cancer, making the contribution of PERK in cancer development complicated.[25] In this regard, a better understanding of the novel physiological impacts of the PERK and what dictates PERK pro-apoptotic signaling from pro-survival signaling has been required to develop cancer therapeutics that target PERK activity. We predict that PERK promotes the ability of cancer cells to adapt to and survive the hostile microenvironment through activation of the metabolic shift toward glycolysis by ATF4 and prevention of apoptosis by Hes1 (Figure 5.1). Aerobic glycolysis has been considered to be fundamental to the transformation of normal cells to cancer cells, and it has been observed that inhibition of PFK or LDH blocks glycolysis, thereby

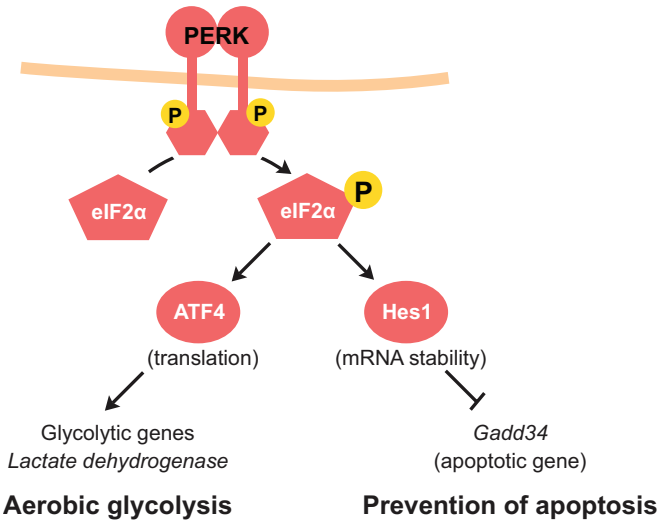

Figure 5.1 Model of the cancer-supporting function of PERK. ATF4 and Hes1 which are activated by the PERK branch of the UPR might contribute to acquisition of cancer hallmarks such as enhanced glycolysis and evasion of apoptosis.

producing anticancer effects.[26] As noted above, expression of both *Pfk* and *Ldh* was increased in our ATF4 overexpression experiment. Therefore, our studies of ATF4 in regulation of aerobic glycolysis will help in understanding tumor metabolism that facilitates cancer cell proliferation. Furthermore, consistent with our finding that Hes1 determines overall cell fate in response to ER stress by acting as a negative regulator of apoptosis, Hes1 has been reported to play an important role in tumorigenesis.[27] In addition to high levels of glycolysis, evasion of apoptosis has evolved to become hallmarks of cancer.[28] In the future, it will be interesting to see if cancer cells showing high levels of Hes1 will have less expression of *Gadd34* and are resistant to apoptosis.

Over the past two decades, the contribution of ER stress to various diseases including cancer, diabetes, and neurodegenerative diseases has been reported. Although the exact direction of the cause and effect relationship between these diseases and ER stress still remains elusive, targeting the UPR has emerged as a promising therapeutic strategy against these diseases. However, further studies are needed to expand our understanding of the UPR signaling pathway itself and its physiological functions to treat diseases driven by ER stress. In this work, I have discovered novel outputs of the UPR beyond the homeostatic control of protein folding and processing which have been the main focus of the UPR. Through these insights and our continued work, we are connecting the UPR to other cellular pathways such as glucose metabolism that does not appear to be directly linked to cellular protein quality control but is important for the cellular responses to ER stress.

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